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Laboratory Manual of Microbiology and Biotechnology

CONTENT

MICROBES: THE WONDERS OF THE WORLD

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Abstract

Microbes are an integral and essential part of the web of life. They carry out a variety of important ecological functions, from recycling organic matter to aiding in the carbon and nitrogen cycles. The microorganisms are nothing but microscopic unicellular organisms include bacteria, fungi, viruses and protozoa. Microorganisms are harmful to us. But it is a fact that microorganisms are useful to us in many ways. Microorganisms help in the production of many food items, making medicines, keeping the environment clean, in manufacturing and in research. Let us learn about microorganisms and its uses in this chapter.

Humans and Microbes: A Brief History

Disease caused by pathogenic microorganisms has been a major killer throughout history. Our species continued survival has been a source of fascination for people all the way back to prehistory. Over the millennia, physicians and scientists have struggled to better understand disease has led to many treatments, some sadly not as effective as others. The domestication of animals for food sources also brought additional problems, with pathogens jumping from animal to human hosts.

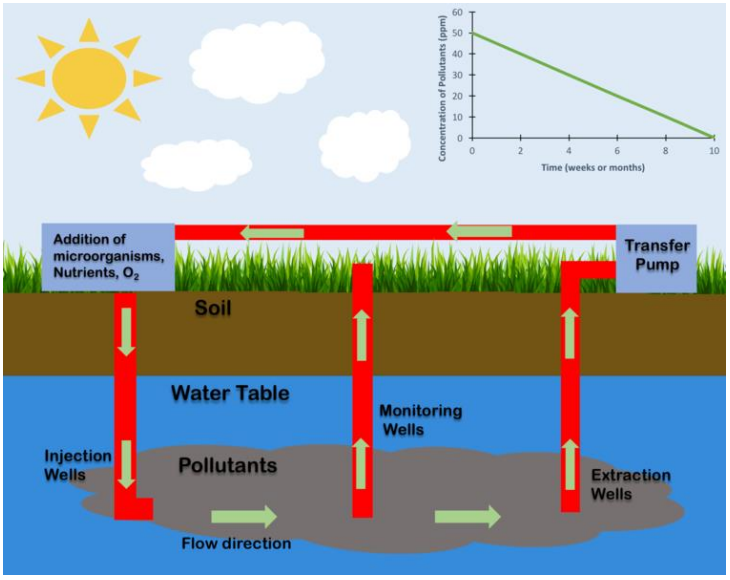
Humans have had an intimate relationship with microbes throughout history, using them for many important purposes. The food industry especially has long used microbes, and over the last couple of centuries they have been utilized in the life sciences and medical industries, the energy industry, waste treatment, and many more industries besides.

Thousands of industrially important products are derived from microbes including actinomycetes, bacteria, and fungi. More products created by microbiologists are entering the market every year.

Microorganisms in nature

Microorganisms play an intrinsic role in almost every natural cycle. Found in most environments, from aquatic to land, air, inside the human body, and even in extreme environments such as hydrothermal vents and volcanoes, microorganisms are an essential part of the web of life.

Microbes help to break down organic matter from plants, animals, and other microbes. They are involved with the nitrogen and carbon cycles. Microorganisms help to generate oxygen and carbon dioxide, as well as fix atmospheric nitrogen into useable forms for multiple organisms. They also help animals ingest food by being part of the gut microbiome. Some species of microbe are symbiotic in nature. It is estimated that the total number of bacteria and archaea on Earth is in the region of 10^{30}.



Microbes display a huge range of metabolic abilities and some are able to degrade or detoxify pollutants, such as petroleum (crude oil) or pesticides, and can be used in **bioremediation**. Some are even able to breakdown plastics.

We urgently need to find viable alternatives to fossil fuels to tackle the problem of climate change. One possibility is the use of photosynthetic microbes (algae and cyanobacteria) to make biofuels. Cyanobacteria and algae can be grown in open ponds or photobioreactors and fed CO_2 and other nutrients to support photosynthesis. The cell components can be extracted to make biodiesel (from lipids) or bioethanol (from carbohydrates, with the help of *Saccharomyces*).

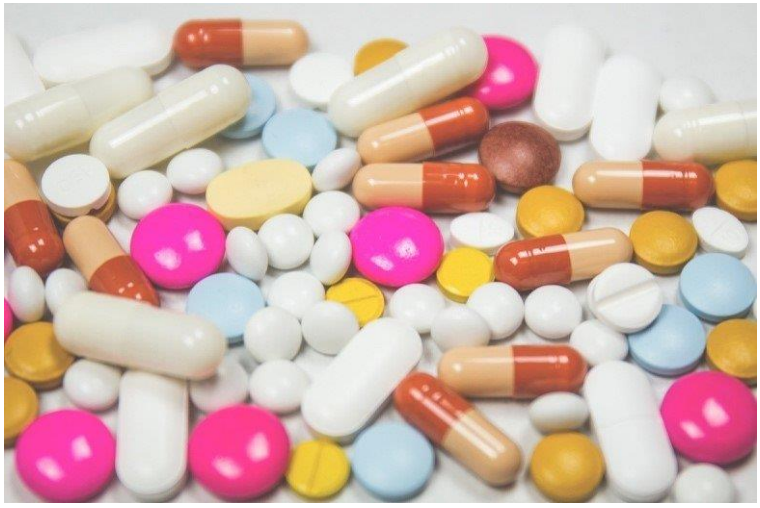
Continued research in the field of microbiology will lead to an even better understanding of how microbes' function and enable us to tackle global threats including climate change, pollution, food shortages, and antimicrobial resistance and emerging infectious diseases.

Microbes and drug discovery

Microorganisms cause numerous diseases in humans, plants, animals, and there are even strains of microbe (such as bacteriophages) that are pathogenic to other microorganisms. Early advances in the field of medicine that utilized microbes include the discovery of penicillin and the development of antibiotics in the early part of the 20th century and the discovery of an effective vaccine against smallpox in the 19th century.

Microbes are used in numerous drug discovery studies today. In 1991, half the pharmaceuticals on the market were either natural products or derivatives. In 1997, 42% of the top-selling pharmaceuticals were obtained from natural sources. Today, hundreds of thousands of secondary metabolites have been identified, and these are used widely in the pharmaceutical industry. Antivirals, antibiotics, and antifungals are commonly used in healthcare settings across the world.

Resistance to drugs has grown in recent decades, most notably antibiotic resistance. This presents several challenges in drug discovery, but new designer drugs are entering the market that is allowing clinicians to treat and cure deadly diseases that are becoming increasingly hard to tackle via existing means.



Biotechnology research has led to the discovery and development of many important **pharmaceuticals** for the treatment of a variety of human diseases and disorders. For example, genetically engineered microbes produce vast quantities of the human hormone insulin, which regulates blood sugar levels and is used to treat diabetes. The human papillomavirus (HPV) vaccine is a structural mimic of the HPV capsid manufactured in genetically engineered *Saccharomyces*. This vaccine protects against several types of cancer caused by high-risk strains of HPV (e.g., HPV-16 and HPV-18 cause 70% of cervical cancers)

Many of the antimicrobials we rely on today to treat infectious diseases were first discovered as natural products of microbes that kill or inhibit the growth of other microbes. The accidental

discovery of penicillin by Alexander Fleming in 1928 and Howard Florey and Ernst Chain finally worked out how to purify penicillin and prove it could be used to treat infections. This ground breaking work marked the beginning of a golden age of antibiotic research and the discovery of many new antimicrobials. Species of *Streptomyces* bacteria were a particularly rich natural source, resulting in the discovery of the antibacterial streptomycin and neomycin and the antifungal nystatin.

Antimicrobials revolutionized the treatment of infectious diseases, but worryingly very few new antibiotics have been introduced since the 1970s and microbes have rapidly evolved resistance to those in clinical use. This has led to a growing interest in alternative treatments, including the potential use of bacteriophages in phage therapy, and prevention strategies, such as biocontrol of insect vectors to limit the spread of diseases such as malaria (you'll find further examples of the use of biocontrol in the section on agriculture below).

A new experimental approach called **gene therapy** is currently under investigation in research laboratories and clinical trials. The technique involves the delivery of nucleic acid (DNA or RNA) into a patient's cells to treat a disease or genetic disorder. Several viruses (eg adenoviruses, retroviruses) have been genetically engineered to act as the delivery vehicle, or vector, for the therapeutic nucleic acids. Diseases that could be treated in this way include certain types of cancer, viral infections (e.g., HIV/AIDS) and inherited genetic disorders.

Microbes in the food industry

The history of microbe use in the food industry stretches back to antiquity. Many food products including bread, yogurt, cheese, kombucha, preserves and preserved meats, and alcoholic beverages take advantage of microbes and their chemical reactions. Microbes also play a vital role in the gut as part of the microbiome, which has spurred techniques to improve the design of microbiome-friendly foods.

Techniques to prevent contamination by pathogenic bacteria improve food safety, design, quality, and shelf-life. Antimicrobial food packaging is a relatively recently developed technology in the food industry. Research into microbes and safeguarding against their danger to human health is of paramount importance to the multi-billion-dollar food industry.

Humans have been exploiting microbes in food production for centuries, long before we knew these organisms, invisible to the naked eye, existed. Lactic acid bacteria (LAB) are used to produce cheese, yoghurt, kefir and kimchi. The budding yeast *Saccharomyces* is used to make bread, beer, cider and wine. Acetic acid bacteria (AAB) are used in traditional manufacturing of vinegar.

Microorganisms are useful in the production of cheese, butter milk and other dairy products.

Cheese:

This is produced from milk and is dependent upon the activity of microorganisms like *Streptococcus lactis, S. cremoris, Leuconostoccitrivorum* etc. The manufacture of cheese consists of curdling the milk by addition of lactic acid bacteria processing of curd to remove moisture, salting and ripening.

During ripening, the microorganism induces proteolytic as well as lipolytic activity. The ripening period of cheese varies from 1 to 16 months. Cheese is a very nutritious food. Its composition is 20-35% protein, 20-30% fat and a small quantity of minerals.

Buttermilk:

The microorganisms used to produce buttermilk are *Streptococcus lactis* or *S. cremoris* and *Leuconostoccitrivorum* or *L. dextranum*. The latter produces volatile acids and neutral products which give the buttermilk its typical flavour.

Butter:

Butter is made by churning the cream which has been soured by lactic acid bacteria.

Production of Alcoholic Beverages:

Alcholic beverages are produced by the fermentation of sugars by yeasts.

$C_6H_{12}O_6 \xrightarrow{\text{Yeast enzymes}} {}_2C_2H_5OH + {}_2CO_2.$

The yeast used is normally *Saccharomyces cerevisae*.

Some common alcoholic beverages are:

Vinegar:

The manufacture of vinegar involves alcoholic fermentation of substrate by yeast. After the fermentation is complete, yeast pulp and other sediments are removed by settling. The supernant is fermented by acetic bacteria, *Acetobacter* sp. The optimum alcoholic concentration is 10-13%.

Beer:

It is prepared from barley malt. Barley malt is converted in to sugar by the enzymatic action of *Aspergillus oryzae*. In next step maltose is converted into alcohol.

Wine:

It is manufactured by the action of yeast, *Saccharomyces ellipsoideus* on the grape juice.

Rum:

It is manufactured by fermentation of molasses with the help of yeast *S. cerevisae*.

Production of Bread making:

Selected strains of *S. cerevisae* are mixed in the dough. Fermentation results in the production of carbon dioxide which causes the dough to rise (leavening) and brings about a desired change in the texture and flavour. The quality of the bread depends upon the strain of yeast, selection of raw materials and incubation conditions.

Production of Food Yeast:

Yeast is used as a very nourishing material and is often taken as a food supplement *Torulopsis utilis* is food yeast cultured and marketed commercially. Yeast is a good source of Vitamin B complex and also rich in proteins. Yeast is a by-product of brewing industry, and is also cultured in a medium containing molasses, cane sugar, potatoes of other fermentable carbohydrates.

Production of Organic Acids:

Some of the common organic acids are produced commercially by microorganisms:

Acetic acid:

It is the vinegar obtained by the fermentation of fruits and sugar containing syrups by *Acetobacter* sp.

Citric acid:

It is an important industrial product obtained through *Aspergillus niger*. Citric acid is used in medicine flavouring extracts, food and candies, manufacture of ink, dyeing etc.

Fumeric acid:

This is produced through *Rhizopus nigricans*.

Gluconic acid:

It is obtained by growing *Aspergillus niger* or *Pencilliumpurpurogenum* in a medium containing corn sugar with ammonium phosphate, magnesium sulphate and calcium carbonate. It is used to produce calcium gluconate, a source of calcium in feeding infants and pregnant women.

Itaconic acid:

It is obtained by growing *Aspergillus terreus* in a medium.

Kojic acid:

It is produced by *Aspergillus flavus* when grown in a medium with sugar plus minerals.

Lactic acid:

It is produced by *Lactobacillus delbruckL. bulgaricus* and *Streptococcus lactis* from corn starch or potatoes Lactic acid is used in confectionery extracts, fruit juices, essences pickles, canned vegetables, fish products etc.

Production of Vitamins:

Microorganisms are useful to mankind as they produce many vitamins. Yeasts manufacture vitamin B. complex. For commercial production of B_{12}, Cobalamin, bacteria and actinomycetes like *Streptomyces olivaceus* and *Bacillus megatherium* are used. Riboflavin (B_2) is produced by a number of microorganisms like *Ashbya gossypii*, *Eremotheciumashbyii* and *Closteridiumbytyricum*. L-Sorbose, a precursor of vitamin C, ascorbic acid, is produced by different species of *Acetobacter* from D-Sorbitol by biological dehydrogenation.

Production of Enzymes:

Many enzymes are synthesized by microorganisms. They are commercially not important as their separation and purification involved high cost.

Some of the microbial enzymes useful to man are:

Amylases:

Produced from *Bacillus subtilis, Aspergillus niger* and *Rhizopus oryzae*.

Dextran sucrase:

Produced from *Leuconostocmeseniteroides*.

Lactase:

Produced by *Saccharomyces cerevisae*.

Lipase:

Obtained from *Candida lipolytica*.

Pectinase:

Manufactured from *Byssochlamysfulvo*.

Proteases and peptidases:

Obtained from *Bacillus subtilis* and *Mortierellarenispora*.

Invertase:

Obtained from *Saccharomycescerevisiae*.

Penicillinase:

Produced from *Actinomyces candidus*.

Waste treatment and environmental remediation:

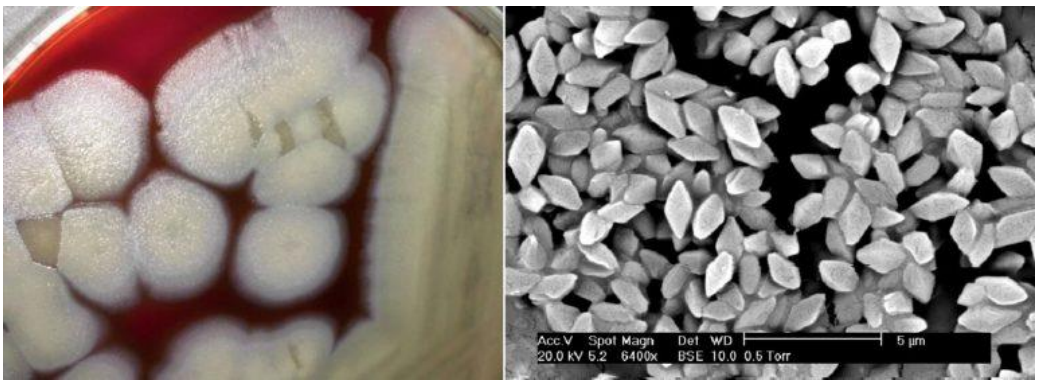
Microbes break down and feed on human waste. They have been used in sewage and wastewater treatment for the last couple of centuries, with ever-more sophisticated applications improving sanitation and health for billions worldwide. Both aerobic and anaerobic bacteria are commonly utilized by the waste industry.

Research over the past few decades has also provided microbial applications for cleaning up pollution and disposing of plastic waste, two of the most critical issues facing modern society. Microbial systems that help to reduce carbon emissions have also been explored extensively.

Microbes and agriculture

Agriculture takes advantage of the natural cycles and behaviour of both plants and animals. Microbes have played a vital role in the history of farming and agriculture. They are a major source of concern for farmers and agricultural scientists due to common diseases such as black rot, bacterial soft rot, bacterial leaf spot, blight, and numerous fungal and viral infections that affect crops worldwide. Numerous treatments and remediation strategies have been developed over the years.

A recent important development in the field of agriculture has been genetic modification. Genetic modification techniques use microbes to amplify beneficial genes. The rise of genetic modification, whilst controversial, offers the possibility of disease-resistant crops and strains that show a more robust response to climate change. Microbes are intrinsically linked with the future of sustainable agriculture.



The human population is growing and we need to produce enough food for everyone. Some microbes negatively impact agriculture by causing diseases in crop plants and livestock but we can use other, beneficial microbes to increase food availability. Several insect pathogens are used as biological pesticides in the **biocontrol** of insect pests that reduce crop yields and limit food production.

Farmers can buy endospores of the bacterium *Bacillus thuringiensis* (Bt) to use as an organic pesticide. Some strains produce crystals in the endospores that are toxic to insects if they eat them.

While controversial, crop plants genetically modified with the Bt toxin genes are resistant to insect pests.

Baculoviruses infect insects and turn them into zombies. If a caterpillar eats leaves contaminated with baculovirus occlusion bodies, occlusion derived virus (ODV) infects cells in the insect gut. The virus replicates by budding from cell to cell, and it makes the infected insect move to the top of the plant towards the light. The baculoviruses produce an enzyme called chitinase which dissolves the insect tissues and turns the caterpillar into a pool of mush. When it rains, occlusion bodies are washed on to the leaves below ready to infect a new host.

Microbes can also be used as **biofertilizers** to improve crop yield. The large-scale cultivation of crop plants rapidly depletes nutrients in the soil and limits plant growth. Nitrogen can be replenished by using legume plants in crop rotation programmes or by the application of free-living nitrogen-fixing bacteria such as *Azotobacter* to the soil. Phosphate-solubilizing bacteria such as *Pseudomonas putida* can be introduced to increase soil phosphates, and plant growth promoting bacteria (eg *Pseudomonas fluorescens*) can be applied to improve the health of crop plants. These biofertilizers help reduce the need for synthetic fertilizers and pesticides, which are produced using fossil fuels that contribute to climate change.

Microbes and aquaculture:

The growth of aquaculture as an industry has accelerated over the past decades; this has resulted in environmental damages and low productivity of various crops. The need for increased disease resistance, growth of aquatic organisms, and feed efficiency has brought about the use of probiotics in aquaculture practices. The first application of probiotics occurred in 1986, to test their ability to increase growth of hydrobionts (organisms that live in water). Later, probiotics were used to improve water quality and control of bacterial infections. Nowadays, there is documented evidence that probiotics can improve the digestibility of nutrients, increase tolerance to stress, and encourage reproduction. Currently, there are commercial probiotic products prepared from various bacterial species such as *Bacillus* sp., *Lactobacillus* sp., *Enterococcus* sp., *Carnobacterium* sp., and the yeast *Saccharomyces cerevisiae* among others, and their use is regulated by careful management recommendations.

Conclusion:

Many innovative technologies are making use of microbes today. They are currently being explored for construction, bioremediation, energy security and generation, and much more. The future of our relationship with these industrially important organisms is likely to be as intricate and intriguing as our long history with microorganisms.

INTRODUCTION TO MICROBIAL TECHNIQUES

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Introduction

Several microbiology techniques and procedures specially developed over decades and decades to study and understand the metabolic processes, genetics, functions, and interaction of microbes with other organisms. The most important methods are techniques for culturing, identification, isolation, staining, and engineering these tiny organisms. These microbes are used to study the prime areas of biological sciences like genetics, plant physiology, evolution, and molecular biology. In addition, some of them contribute to better our health, but some cause life-threatening diseases. Others are used in food and beverage production, and to understand all this, we need to study these organisms.

The microbiology techniques are broadly classified into 3 types based on the type of experiments

1.1. Culturing and Aseptic Techniques

1.2. Bacteria enumeration

1.3. Identification of Pathogens

1.4. Application in agriculture

1.1.1. Aseptic Techniques

To study specific organisms, it's mandatory to grow them in a controlled lab environment. A complete sterile condition protects the pure microbial culture from contamination by other organisms entering through the air, water, or other unsterile sources. Some techniques used in labs to maintain a completely aseptic environment include.

i. Sterilization: It's the complete removal of all other microbial forms, including viruses, bacteria, fungus, spores, and other vegetative cells from the surface or the culture media.

Based on the purpose of the sterilization, the method is categorized into two groups:

a. Physical Methods: Killing of contaminants or microbial forms using heat, sunlight, drying, filtration, or irradiation techniques (**e.g.,** UV, infrared, gamma radiation and X-ray).

b. Chemical Methods: Chemicals such as phenol (and any other related compounds), dyes, soaps, detergents, alcohol, gaseous compounds, and heavy metals and their compounds to destroy microorganisms.

ii. Disinfection: Disinfection is the process of killing microbes or inhibiting their growth from inanimate objects or surfaces by using physical or chemical agents like phenol, chlorine, alcohol, and heavy metal and their compounds.

iii. Sanitization: Complete elimination of all pathogenic and non-pathogenic microbes from surface tops to reduce contamination. It's also employed in daily lives to sanitize hands or in restaurants, dairies, and breweries to remove microbes and prevent infection and contamination.

1.1.2. Culturing Techniques:

i. Inoculation: It's a basic technique used in microbiology labs to place microbial cultures onto a culture medium. It's performed using an apparatus, called inoculation loop. It's mainly used in streaking and culture plate techniques. The small sample picked up and transferred from the culture is known as inoculum.

ii. Isolation: Isolation is a microbiological technique in which a specific microbial strain is isolated from a mixed culture of microorganisms by culturing the microbes on a selective culture media. However, the procedure must be repeated several times to eliminate contamination by other microbes and achieve a pure culture of the microbial strain, which is then observed in culture plates as discrete/isolated individual colonies.

1.1.3. Culturing techniques: Microbes are grown in labs on culture media, which supply their nutritional requirements. These requirements vary for different microorganisms;thus, a spectrum of culture medium recipes have been developed by scientists to obtain the desired microbial strain.

a. Simple or basal media: It consists of sodium chloride, peptone, meat extracts, and water, for example, Nutrient Broth.

b. Complex media: This contains an additional special ingredient that helps to enhance a special characteristic or provide nutrients for the growth of certain microbes. It may contain extracts from plants, animals, and yeast, such as blood, yeast extracts, serum, milk, meat extracts, soybean digests, and peptone.

c. Synthetic or defined media: It's used for research purposes. They are prepared by following an exact formula and mixing distilled water with specific amounts of inorganic and organic chemicals.

d. Special media: The basic medium supports the growth of a broad spectrum of microbial forms. However, a special growth condition is required for the culture and isolation of only a certain type or selected strain of bacteria. These formulated media to grow a microorganism chosen are known as special media. It's further categorized into different groups.

e. Selective media: It inhibits the growth of selected microorganisms while allowing the other to flourish. Examples include desoxycholate citrate medium for dysentery bacilli or mannitol salt agar containing 7.5% NaCl for *Staphylococcus.*

f. Enriched media: It contains complex organic substances like hemoglobin, serum, blood, or growth factors to support the growth of certain microbes. Examples are blood agar (widely used to grow certain streptococci and other pathogens) and chocolate agar.

g. Indicator media: It contains an indicator that changes color when a certain bacterium grows on the medium. For example, the addition of sulfite in the Wilson and Blair medium changes color to black when Salmonella typhi colonies grow on the medium.

h. Differential media: This media allows the growth of different bacterial species and distinguishes them based on their size, shape, color, or formation of gas bubbles or precipitates in the medium. Examples are MacConkey medium and blood agar.

i. Transport media: It's a buffer solution containing peptone, carbohydrates, and other nutrients (except growth factors) to maintain the viability of the bacteria during transport without allowing their multiplication. An example is the Stuart medium for gonococci.

g. Anaerobic media: It contains ingredients that support the growth of anaerobic bacteria. An example is Robertson's cooked meat media.

1.1.4. Common culture techniques

i. Streak plate method: In this technique, an inoculation loop is dipped in a diluted microorganism suspension and streaked on the solid surface of the culture medium. The plate which gets streaked is known as the streak plate. The method is generally used to obtain individual bacteria colonies from a concentrated suspension or to prepare a pure culture of a bacterial strain.

ii. Spread plate method: In this method, a very small volume of the liquid suspension of the microorganism is poured on the solidified surface of the media-containing plate. Then, an L-shaped glass rod is used to spread the liquid evenly all over the plate surface. This is performed to obtain individual colonies of the microorganisms but can also count the number of the microbial population.

iii. Pour plate method: In this technique, a serially diluted microorganism suspension is pipetted in a sterile Petri dish. Then, a liquified, cooled culture media is poured into the plate. After the media is solidified, the culture plate is incubated for specific bacterial growth. It's performed to estimate the viable bacterial count in a microbial suspension.

1.1.5. Incubation

After the microorganisms are inoculated in plates, the culture plates are sealed from base to lid using adhesive tape to prevent contamination. Then, the plates are kept in the incubator for the required time and temperature for the growth of the organisms. Furthermore, keeping the plates in an inverted position prevents the formation and fall of water droplets into the culture media. If it's required to store the microbial samples for later experiments, the following storage techniques are used:

i. Refrigeration ii. Deep freezing

iii. Lyophilization iv. Freezing in liquid nitrogen

1.2. Bacteria Enumeration:

Counting microbial colonies is an essential task in performing a range of experiments. Here are some enumerating techniques

i. Serial dilution: It's used to lower the concentration of bacteria to a required amount for the purpose of experimentation to culturing processes.It helps to count the number of microbial populations and perform experiments with the necessary number of microbial populations.

ii. Plate counts: By applying the plate count method, it is possible to determine how many microbial colonies could potentially emerge under the given physical and chemical conditions, such as pH, temperature, available nutrients, and growth inhibitory compounds.

iii. Most probable number (MPN): In this statistical technique, a broth is inoculated in a 10-fold dilution, predicting the number of viable microorganisms per volume in a given sample.

iv. Using spectrophotometer: A spectrophotometer is used to estimate the growth of microorganisms in the culture with respect to time or at a certain time.

1.3. Identification of Pathogen or microbes:

Pathogen identification is important for several applications. it's used to know which microbe is involved in contamination and food spoilage, which has useful applications in human lives, and which microorganism caused the particular disease for correct diagnosis and treatment in medicine

Microbes are identified by

i. Morphology: This is the first step in the identification process where the microorganisms are assessed with the naked eye based on colony texture, shape, and size.

ii. Staining techniques: The identification is done by staining microbes with certain chemicals and observing them under a microscope to assess their cell properties.

a. Simple staining: In this method, bacteria are stained using a single reagent and identified based on their size, shape, and arrangement of cells.

b. Differential staining: Here, at least three chemical reagents are used to stain bacteria, and identification is done based on the color the microorganism shows.

c.Gram staining: Here, certain chemical reagents are used to differentiate two principal groups of bacteria, gram-positive and gram-negative.

iii. Microscopy techniques: Some microorganisms like viruses cannot be identified using a simple microscope, as in the case of other microorganisms. Therefore, a high-throughput electron microscope is required to identify them.

iv.Biochemical tests: Different tests, such as oxidase test, catalase test, indole test, and Dnase test are performed to differentiate microorganisms based on their enzymatic activities.

v. Motility: The motility capacity of microorganisms is assessed to distinguish them from other microorganisms and identify their groups.

vi. Microbial serology: It's a test performed by using methods like Enzyme-linked immunosorbent assays (ELISA), western blotting, agglutination, and direct and indirect immunofluorescence to determine the bacterial or viral antibodies and antigens.

vii. Molecular analysis: DNA based sequencing are used to identify, characterize, and classify microorganisms

1.4. Application of different types of microbial biofertilizers

a.Nitrogen biofertilizer application:

- Rhizobium for legume crops.

- *Azotobacter* and *Azospirillum* for non-legume crops.
- *Acetobacter* for sugarcane only.
- Blue-green algae (BGA) and Azolla for low-land paddy.
- Frankia for Casuarina and Alnus.

b. Phosphorus biofertilizer application:

PSB can be used for all crops, including paddy, millets, oilseeds, pulses and vegetables.

The methods recommended for application are:

i. Seed treatment;

ii. Seedling dipping;

iii. Soil application.

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STERILIZATION TECHNIQUES

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Introduction:

Any procedure known as sterilization eliminates, or renders inactive all life forms, particularly microorganisms like fungi, bacteria, spores, and unicellular eukaryotic organisms, as well as other biological agents like prions, that are present in or on a particular surface, object, or fluid.

1. Wet Heat (Autoclaving)

- Most laboratories use autoclaving, which involves heating the material to be sterilized with pressurized steam.
- This is an extremely efficient procedure that eliminates all bacteria, spores, and viruses, albeit certain particular bugs require particularly high temperatures or long incubation periods.
- By hydrolyzing and coagulating biological proteins, which is effectively accomplished by extreme heat in the presence of water, autoclaving kills bacteria.
- The steam is what generates the tremendous heat. At 100^{o}C, pressurized steam has a large latent heat; it can store seven times as much energy as water.
- This heat is released as it comes into touch with the item to be sterilizer's cooler surface, enabling for quick heat delivery and good penetration of dense materials.

2. Dry Heat (Flaming, Baking)

- One significant distinction separates dry heating from autoclaving.
- Dry heat, on the other hand, typically oxidizes biological components to kill microorganisms.
- Higher temperatures are needed for effective sterilizing by dry heat since this consumes more energy than protein hydrolysis.
- For instance, autoclaving at 121^{o}C may often sterilize in 15 minutes, whereas dry heating typically requires 160^{o}C to sterilize in the same length of time.

3. Filtration

- Filters function by forcing a solution through a filter with tiny pores that prevent germs from passing through.
- Filters can be built of heat-fused glass particles in the form of sintered glass funnels or, more frequently today, cellulose esters in the form of membrane filters.
- Filters having an average pore diameter of 0.2um are typically employed for the elimination of microorganisms.

4. Solvents

- Although isopropanol is a better solvent for fat and is probably a better choice, ethanol is frequently used as a disinfectant.
- Both solvents must be diluted to between 60 and 90 percent in water in order to function because they denaturize proteins through a process that needs water.
- Ethanol and IPA are effective at eliminating microbial cells, they have no impact on spores.

5. Radiation

- Electromagnetic radiation such as ultraviolet (UV), x-rays, and gamma rays all have severely destructive effects on DNA, making them great sterilizing agents.

- Their penetration is the primary distinction between them in terms of their efficacy. Only a relatively small region around the bulb is sterilized because UV has a limited ability to penetrate air.
- It is quite helpful for sterilizing tiny spaces, such laminar flow hoods, and is generally safe.
- As a result of their greater penetration, X-rays and gamma rays are more lethal but also particularly effective for mass cold sterilization of plastic products (such as syringes) during manufacture.

6. Gas Sterilization

- Equipment that is susceptible to heat or moisture can be sterilized using ethylene oxide, which is frequently done with medical equipment like catheters and stents.
- By alkylation, ethylene oxide effectively stops cell metabolism and replication. Equipment must be aerated to remove any residue after sterilization because ethylene oxide is rapidly absorbed.
- In addition to being extremely poisonous, ethylene oxide poses a number of health hazards.

PURE CULTURE TECHNIQUES

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Introduction:

In microbiology, pure culture is a laboratory culture containing a single species of organism. A pure culture is usually derived from a mixed culture (one containing many species) by transferring a small

sample into new, sterile growth medium in such a manner as to disperse the individual cells across the medium surface or by thinning the sample many folds before inoculating the new medium.

Isolation of a pure culture may be enhanced by following the aseptic techniques and providing a mixed inoculum with a medium favouring the growth of one desired organism over the others.

Aseptic Techniques

Aseptic techniques (also called sterile techniques) are defined as the processes required for transferring a culture from one vessel to another without introducing any additional organisms to the culture or contaminating the environment with the culture. It is necessary to sterilize all the essential materials like laminar air flow chamber, medium, petriplate, glass rod, inoculation loop etc., to obtain the pure culture.

Culture media

General, Selective and Differential Media

In addition to using sterile technique, utilizing the differential and selective media increase the possibility of getting pure culture.

A general medium facilitates the growth of all type of microbes. Eg. Nutrient Agar for bacteria

A selective medium "selects" for the growth of specific microbes (while inhibiting the growth of others) by virtue of some distinguishing nutritional or environmental factors (e.g., ability to utilize lactose as the sole carbon source, survival at a low or high pH, presence of selective inhibitors such as bile, crystal violet, antibiotics). Eg., MacConkey Agar (MAC): This is used to isolate Enterobacteriaceae and other related enteric Gram-negative rods. Included bile salts and crystal violet in the medium inhibit the growth of Gram-positive bacteria

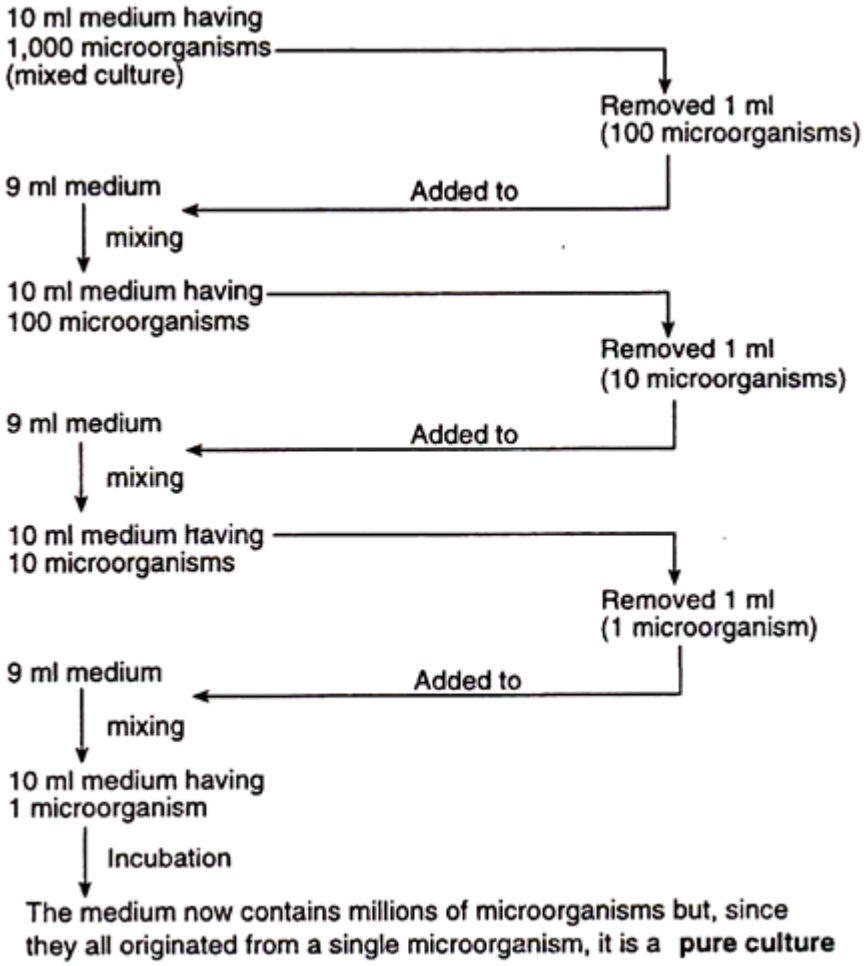
A differential medium enlists a particular bacterial property to allow visual differentiation of one organism from another (e.g., ability to ferment a particular carbohydrate like lactose alters a pH indicator and a lactose-fermenter colony has a distinctive color compared to lactose non-fermenters which are not colored). Eg., Mannitol Salt Agar (MSA): Mannitol-fermenting bacteria produce a yellow zone surrounding their growth while other *staphylococci* do not produce a color change.

Methods to isolate pure culture

1. Serial Dilution Method:

This is the method commonly used to obtain pure cultures of those microorganisms that have not yet been successfully cultivated on solid media and grow only in liquid media.

A microorganism that predominates in a mixed culture can be isolated in pure form by a series of dilutions. The inoculum is subjected to serial dilution in a sterile liquid medium, and a large number of tubes of sterile liquid medium are inoculated with aliquots of each successive dilution.



2. Streak Plate Method:

This method is used most commonly to isolate pure cultures of bacteria. A small amount of mixed culture is placed on the tip of an inoculation loop/needle and is streaked across the surface of the agar medium. The successive streaks "thin out" the inoculum sufficiently and the micro-organisms are separated from each other.

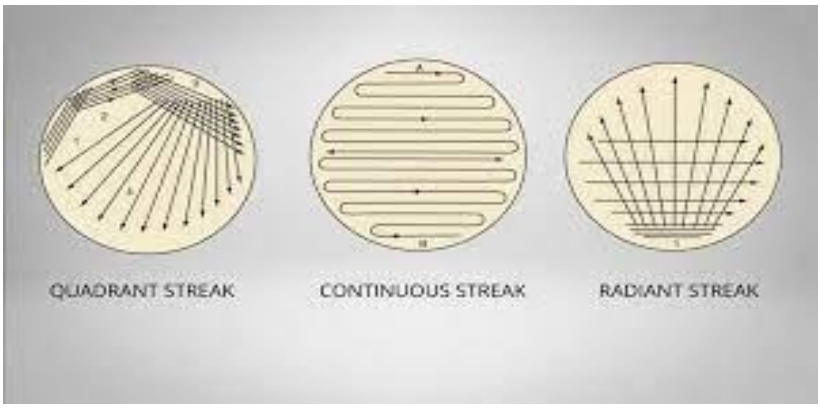
Types of Streaking

Quadrant Streaking

It is the most commonly used and the most preferred method where four equal-sized sections of the agar plate are streaked. It is also referred to as the "four-quadrant streak" or "four sectors" or "four-way streak" method.

In this method, each plate is divided into four equal sectors and each adjacent sector is streaked sequentially. The sector which is streaked first is called the first sector or the first quadrant, and it has the highest concentration of inoculum. Gradually the second, third, and fourth quadrants will have

diluted inoculum. By the time the fourth quadrant is streaked, the inoculum is highly diluted giving rise to isolated colonies following the incubation.



Continuous Streaking

It is another commonly followed method where an inoculum is evenly distributed in a single continuous movement from starting point to the center of the plate. There is no need to divide the plate and sterilize the loop during the process. It is easy and quick; we can use it only for the diluted inoculum.

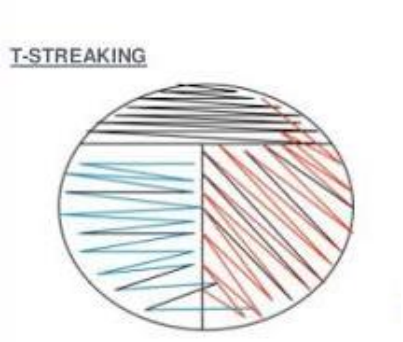
Radiant Streaking

It is another method of streaking where the inoculum is first streaked at one edge and spread in vertical lines above the edge. Finally, the vertical lines are cross streaked diagonally.

T-Streaking

It is another method of streaking where the agar Petri plate is divided into three sections and each section is streaked. Hence, this method is also known as the “three-sector streak” method.

The media is divided into three sections by drawing a letter “T” and each adjacent section is streaked



sequentially. By the time the final section is being streaked, the inoculum is diluted to the point to give rise to isolated colonies following the incubation.

Zigzag Streaking

It is another form of continuous streaking where a loopful of the specimen is streaked all over the plate in a zigzag pattern in a single continuous movement. It is commonly done to propagate the pure culture and culture them in large quantities.

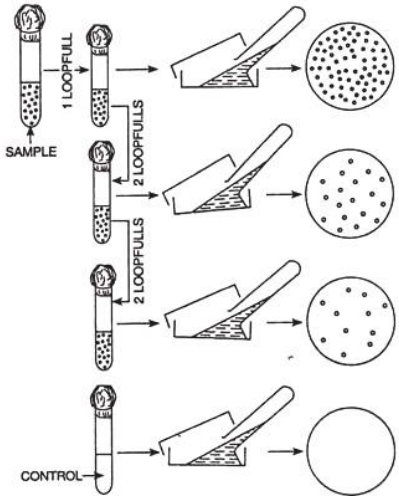
3. Pour Plate Method:

This method involves plating of diluted samples mixed with melted agar medium. The main principle is to dilute the inoculum in successive tubes containing liquefied agar medium so as to permit a thorough distribution of bacterial cells within the medium.

The contents of each tube are poured into separate Petri plates, allowed to solidify, and then incubated. When bacterial colonies develop, one finds that isolated colonies develop both within the agar medium (subsurface colonies) and on the medium (surface colonies). These isolated colonies are then picked up by inoculation loop and streaked onto another Petri plate to insure purity.

4. Spread Plate Method

In this method, the mixed culture or microorganisms is not diluted in the melted agar medium (unlike



the pour plate method); it is rather diluted in a series of tubes containing sterile liquid, usually, water or physiological saline.A drop of so diluted liquid from each tube is placed on the center of an agar plate and spread evenly over the surface by means of a sterilized bent-glass-rod. The medium is now incubated.

STORAGE AND PRESERVATION OF MICROORGANISMS

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Introduction:

Preservation of microorganisms for future study has a long tradition in microbiology. Culture collections of microorganisms are valuable resources for scientific research in microbial diversity and evolution, patient care management, epidemiological investigations, and educational purposes. Preserved individual strains of microorganisms serve as permanent records of microorganisms' unique phenotypic profiles and provide the material for further genotypic characterizations. Effective storage involves maintaining an organism in a viable state free of contamination and without changes in its genotypic or phenotypic characteristics. The organism must also be easily restored to its condition prior to preservation.

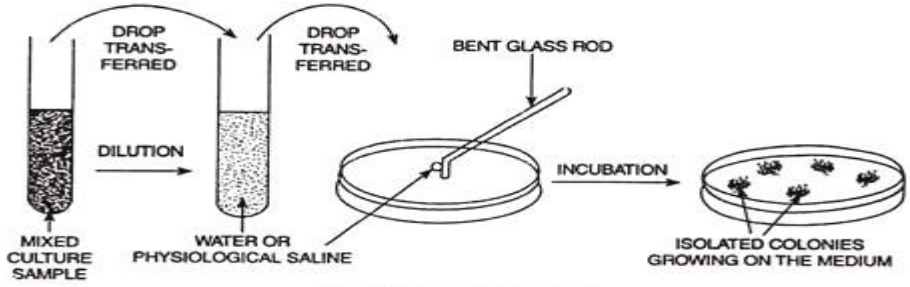
Microbiological laboratories concerned with microbial studies and to preserve cultures for a short period or manyyears to conserve and to retain all the characteristics of the organisms. These preserved cultures may be made available infuturefor variouspurposessuch as:

- Useinthelaboratoryclasses
- Researchwork
- Useastestagentsforparticularprocedure

Somemethodsusedforculturepreservationincluderefrigeration,deepfreezing,freezingunderliquid nitrogen and lyophilization.

Refrigeration

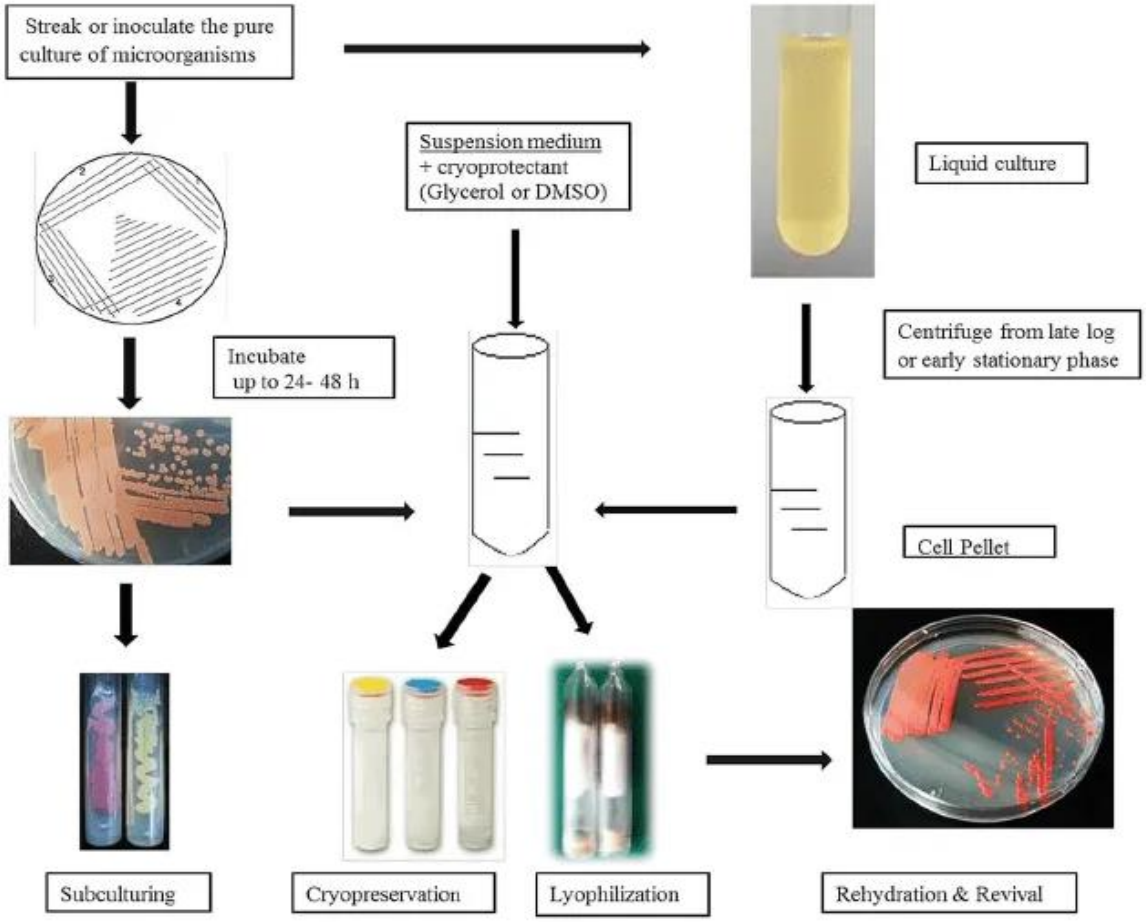
Live cultures on a culture medium can be successfully stored in refrigerators or cold rooms



maintained at4ºC.Generally,themetabolicactivitiesofthemicroorganismswillbegreatlysloweddownatthistemperature. Storing cultures in a refrigerator at a temperature of 4ºC, slows growing protects from damagedue

to evaporation of medium and preserve the culture. Thus, growth will occur slowly, nutrients will beutilized and waste products produced, which will eventually kill the microorganisms. So, subculturing ofrefrigerated cultures is to be carried out at regular intervals. In the case of bacteria, subculturing should bedone at intervals of 2-3 weeks. In the case of fungi, regular subculturing is necessary at intervals of 3-4months.

Different Methods of Maintenance and Preservation of Microorganisms



DeepFreezing

Culturescanbepreservedforseveralyearsinglycerol stock inadeepfreezer.Inthismethodapproximately 2 ml of the glycerol solution is added onto the agar slope culture by shaking. The culturesuspension is transferred into each ampoule which is placed in a mixture of industrial methylated spirit andCO_2andisfreezedrapidlyto-70ºC.Ampoulesareremovedfromthemixtureandplaceddirectlyintoadeep freezer at 40ºC. During transfer from these stock cultures, tubes are placed to water bath at 45ºC for afewsecondsor untilthesuspensions meltand areaseptically streaked ontoagarplates.

FreezingunderLiquidNitrogen

Freezing in liquid nitrogen at temperature of -196ºC also suspends metabolism of cells and these surviveunchanged for long periods. In this method, cell suspension in the presence of a

stabilizing agent such asglycerol or dimethyl sulfoxide, that prevents the formation of ice crystals which may kill frozen cells, issealed into small ampoules and stored in liquid nitrogen refrigerator. Most species of bacteria can remainviable for 10-30 years or even more without undergoing change in their characteristics. The liquid nitrogenmethodhas been successfulwith manyspeciesthatcannotbepreserved bylyophilization.

Lyophilization

Lyophilization or freeze drying is the rapid dehydration of organisms while they are in a frozen state. Mostof the microbes are protected from the damage caused with water loss by this method. Because metabolismrequires water, the organisms are in a dormant state and can retain viability for over 30 years unchanged intheir characteristics. In this technique, the culture is rapidly frozen at -70ºC and then dehydrated by vacuumandthetubescontainingfreezedried culturesaresealed andstoredin thedarkat4ºCinrefrigerators. It is the most satisfactory method of long-term preservation of microorganisms. It's universally used for thepreservation of bacteria, viruses, fungi etc. Lyophilized cultures are revived by opening the vials addingliquidmediumand transferringthecultureto asuitable growth medium.

MICROBIAL PIGMENTS FOR THERAPEUTIC APPLICATIONS

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Pigment

A pigment is a material that changes the colour of reflected or transmitted light as the result of wavelength-selective absorption. In other words, it's a substance that appears a certain colour because it selectively absorbs certain wavelength of light.

The primary purpose to use pigments is to provide colour to materials, whether they are textiles or paints. Some people often consider pigments and dyes to be the same but they're quite different. The major difference between the both is in terms of their solubility.

While a dye can dissolve by itself in a liquid, pigments can be dissolved in a liquid with the help of a binder. Dyes are primarily applied in the textile and paper industry, pigments are used in industries such as colouring paints, inks, cosmetics, and plastics.

Types of pigments

Based on the method of their formulation, pigments can be categorized into two types: inorganic pigments and organic pigments.

Organic Pigments

This type of pigments occurs naturally and they've been used for centuries. They're quite simple in their chemical structure. They're named organic as they contain minerals and metals that give them their colour. Organic pigment manufacturers produce them through a simple process that's made of washing, drying, powdering and combining into a formulation.

Compared to inorganic pigments, these usages of these pigments are less frequent and that's why there are limited number of organic pigment suppliers. These pigments are used when the required colour strength isn't too much.

Inorganic Pigments

As you can guess by its name, this type of pigments is exactly the opposite type of organic pigments. These pigments are also known as "synthetic pigments." They're formulized in labs and offer great scope of control to inorganic pigment manufacturers. Inorganic pigments are manufactured by relatively simple chemical processes such as oxidation.

Inorganic pigment suppliers supply this type of pigments mainly to paints, plastics, synthetic fibres and ink industry. In uses where bright colours are required, organic pigments are used as they bolster

high colour strength. Inorganic pigments include white opaque pigments which are commonly used to lighten other colours and also to provide opacity.

Examples of Pigments

Carotenoids

Astaxanthin

β-Carotene

Canthaxanthin

Melanin

Prodigiosin

Phycocyanin

Riboflavin

Violacein

Microbial pigments for therapeutic applications.

Microorganisms are the most flexible instruments of biotechnology for creating different molecules including pigments, enzymes, and organic acids and antibiotics. A broad variety of microorganisms including bacteria, yeast, molds, and algae are able to produce pigments. This group of microorganisms must satisfy several criteria, such as (1) able to use a varied range of C and N sources; (2) able to tolerate a wide variety of utmost conditions like pH, temperature, the concentration of minerals, etc.; (3) nontoxic and non-pathogenic; (4) a good amount of pigment yield; and (5) easier separation from its cell mass.

Bacteria

A diverse range of pigments are produced by bacteria such as actinorhodin, pyocyanin, carotenoids, melanin, canthaxanthin, prodigiosin, violacein, zeaxanthin, etc., and possesses certain advantages over fungi and other organisms such as a reduced life cycle making it easy to engineer genetically. It can be pervasive and are present in different niches like freshwater, soil, desert sand, and marine samples. In comparison to other bacterial communities, various genera of Actinobacteria such as *Micromonospora, Streptomyces, Actinomadura, Nocardia, Kitasatospora, Microbispora, Actinoplanes, Streptosporangium, Rhodococcus*, and *Thermomonospora* are stated for the production of a broad range of pigments. Bacterial pigments offer certain advantages such as it has a broad range of activities, pigment-associated genes can be manipulated easily, simple techniques for extraction with minimum operating costs.

Fungi

Filamentous fungi are gaining industrial importance as it can be easily grown in the laboratory and can also enhance large scale production. A varied range of pigments are produced by filamentous fungi like carotenoids, phenazines, violacein, melanins, etc. Genera like *Curvularia, Drechslera, Eurotium*, and *Fusarium* were reported to produce pigments like anthraquinones, chrysophanol, cynodontin, tritisporin, naphthaquinoneserythroglaucin, flavin, helminthosporin, and dihydroxy

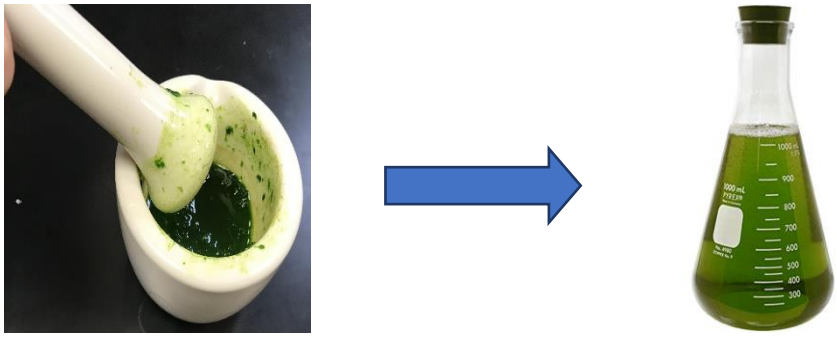
naphthalene melanin. *Monascus* used for the production of ang-kak (red mold rice) has been reported to be the most ancient use of fungal pigment. Various colored pigments such as yellow (monascine) orange (monascorubrine rice), purple (monascorubramine) has been produced by Monaseus and are used in different food items around the globe. Beyond 200 species of fungus have been noted for the synthesis of carotenes among which mostly belonging to the zygomycetes; order Mucorales including *Blakeslea*, *Phycomyces*, and *Mucor*.

Algae

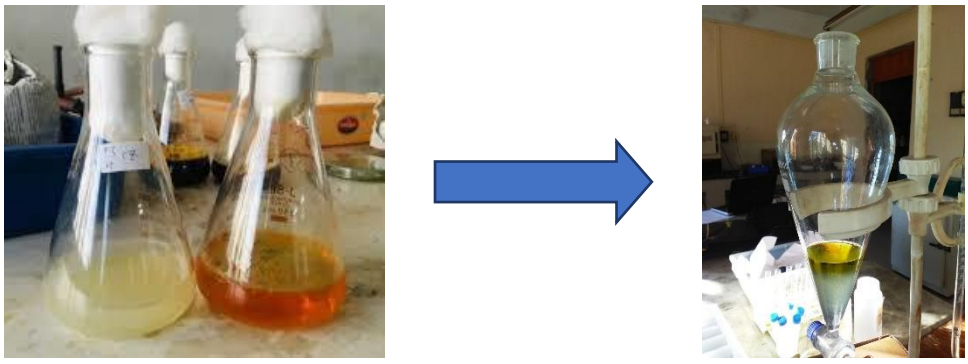
Various types of pigments like canthaxanthin, astaxanthin, lutein, β-carotene are produced by Algae. *Dunaliella salina*, belonging to class Chlorophyta produces β-carotene under high saline and high light intensity conditions. Apart from main chlorophyll pigments, *Rhodophyta*, another red microalga, are also known to contain both red phycocyanins and blue phycoerythrin pigments. Such red and blue pigments account for approximately 30% of the total algal cell protein.

Pigment Extraction from Plant/ Microbes

Plant extraction is a process that aims to extract certain components present in plants. It is a solid/liquid separation operation: a solid object (the plant) is placed in contact with a fluid (the solvent). The plant components of interest are then solubilized and contained within the solvent. The solution thus obtained is the desired extract.



Pigment produced by the microbes can be isolated using solvent extraction. These pigments can be further purified and characterized for physical and chemical characteristics using various instrumental-based analytical techniques



Extraction Methods

A mixture is composed of two or more types of matter that can be present in varying amounts and can be physically separated by using methods that use physical properties to separate the components of the mixture, such as evaporation, distillation, filtration and chromatography.

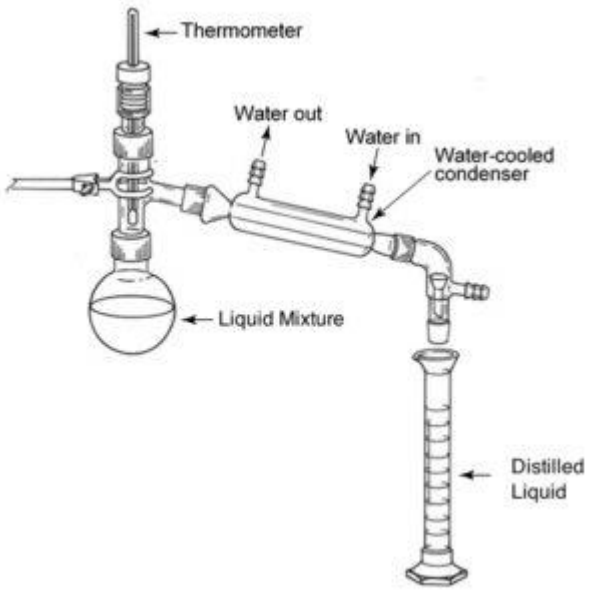
Evaporation can be used as a separation method to separate components of a mixture with a dissolved solid in a liquid. The liquid is evaporated, meaning it is converted from its liquid state to gaseous state. This often requires heat. Once the liquid is completely evaporated, the solid is all that is left behind.

Evaporation can be used as a separation technique.

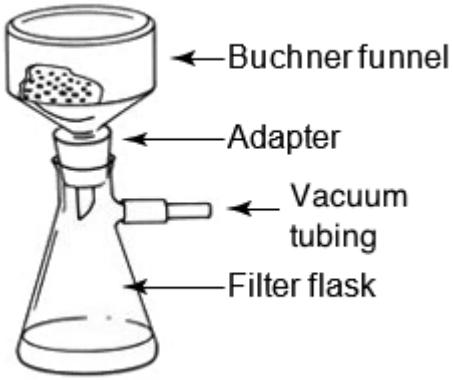
Distillation is a separation technique used to separate components of a liquid mixture by a process of heating and cooling, which exploits the differences in the volatility of each of the components.

Distillation procedure: 1) the round bottom flask contains the liquid mixture which must be heated to a vigorous boil, 2) the component with the lower boiling point will change into its gaseous state, 3) upon contact with the water-cooled condenser, the gas will condense, 4) trickle down into the graduated cylinder where the chemist can them recuperate the final distilled liquid, and 5) the other liquid component remains in the round bottom flask.

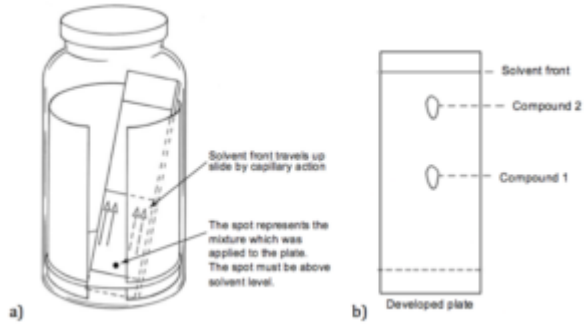
Distillation apparatus.



Filtration is a separation technique used to separate the components of a mixture containing an undissolved solid in a liquid. Filtration may be done cold or hot, using gravity or applying vacuum, using a Buchner or Hirsch funnel or a simple glass funnel. The exact method used depends on the purpose of the filtration, whether it is for the isolation of a solid from a mixture or removal of impurities from a mixture.



Filtration apparatus.Filtration procedure: 1) the mixture is pored through a funnel lined with a filter paper, 2) the filtrate (liquid) drips through to the filter flask, 3) the solid remains in the funnel.Though **chromatography** is a simple technique in principle, it remains the most important method for the separation of mixtures into its components. It is quite versatile for it can be used to separate mixtures of solids, or of liquids, or mixtures of solids and liquids combined, or in the case of gas chromatography, can separate mixtures of gases. The two elements of chromatography are the stationary phase and the mobile phase. There are many choices of stationary phases, some being alumina, silica, and even paper. The mobile phase, in liquid chromatography, can also vary. It is often either a solvent or a mixture of solvents and is often referred to as the eluant. A careful choice of eluting solvent helps to make the separation more successful. The mixture is placed on the stationary phase. The eluant passes over the mixture and continues to pass through the stationary phase carrying along the components of the mixture. If a component in the mixture has greater affinity for the mobile phase (eluant) than the stationary phase, it will tend to be carried along easily with the eluant. If another component in the mixture has a greater affinity for the stationary phase than the mobile phase then it will not be carried along so easily. A separation is thus obtained when



the different components in a mixture have different affinity for the stationary and mobile phase. Three important types of chromatography based on the principles discussed above are: 1) thin layer chromatography (TLC), 2) column chromatography, and 3) gas chromatography.

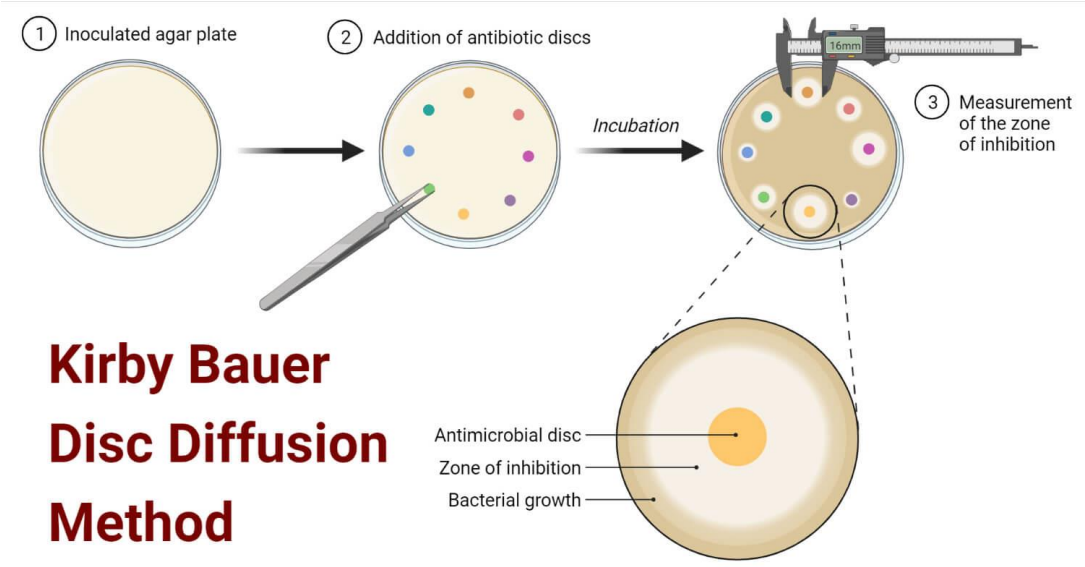
Thin layer chromatography is a one type of chromatography. a) The stationary phase can be a thin film of alumina or silica on glass or even paper. The plate is placed in a developing tank which contains the mobile phase (eluant) which travels up the plate by capillary action. b) A separation is obtained because the component of the mixture that has a stronger affinity for the eland (compound 2) travels faster up the plate, than the component that has a strong affinity to the stationary phase (compound 1).

Biological activity

Antimicrobial Assay

Kirby Bauer Disc Diffusion Method

1. Sterilize the area with disinfectant and open burner before performing the test.
2. A sterile cotton swab is dipped into the inoculum and remove excess medium by pressing the swab onto the wall of the tube.
3. Swab the surface area of the plate completely by rotating the plate. This technique is called lawn culture or carpet culture.
4. Allow the plates to dry for 5 minutes so that the medium absorbs the inoculum properly.
5. Firstly, sterilize the forceps with alcohol before picking up antibiotic discs.
6. Discs should be placed at a distance of 24mm.
7. Lightly touch each disc with forceps to ensure that it is in good contact to avoid misplacement.
8. Incubate the plate upside down for 24 hours at 37ºC.



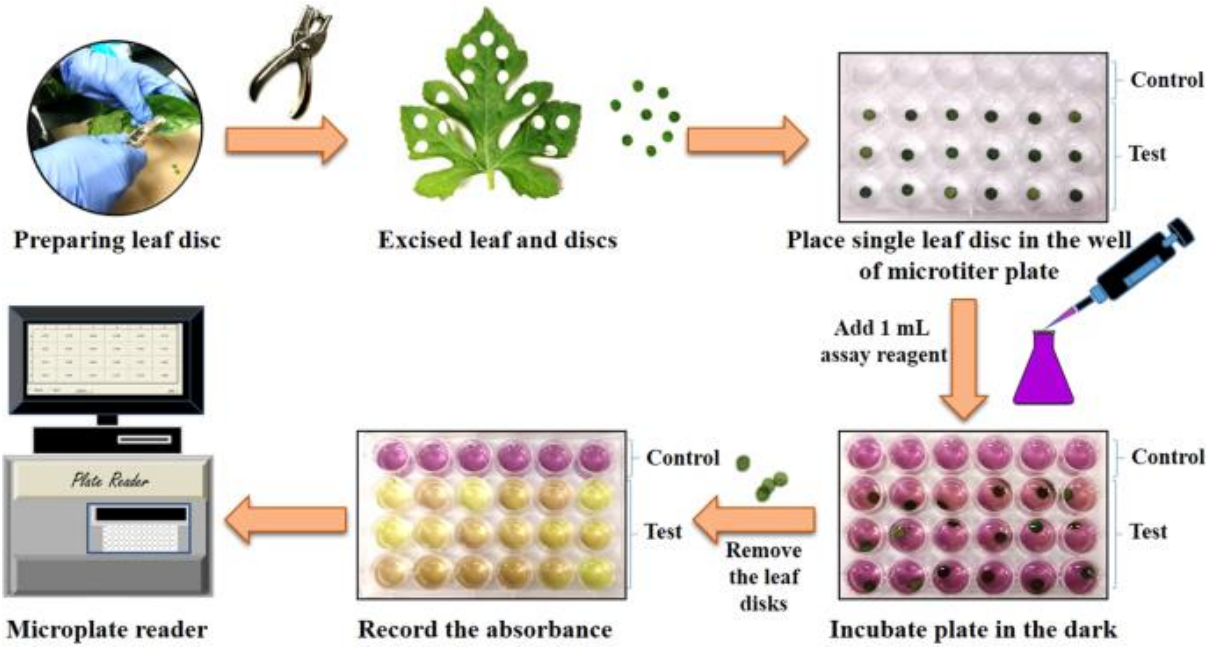
Antioxidant Assay

DPPH assay

1. 0.5 ml of the fraction solutions (10, 20, 40, 60, 80, 100, 200, and 400 µg/ml in ethanol) will be added to 0.5 ml of a DPPH solution (0.1mM in ethanol).
2. Incubate for 30 min at room temperature.
3. Measure the absorbance at 517 nm.
4. The free radical scavenging activity of each fraction will be determined by comparing its absorbance with that of a blank solution (no sample).
5. Ascorbic acid is used as standard.
6. The ability to scavenge the DPPH radical was calculated using the following equation:

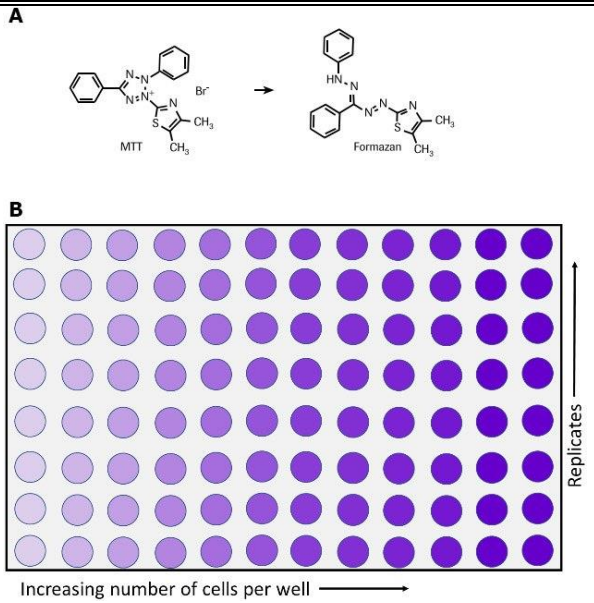
DPPH scavenging activity (%) = [(Ac – At) /Ac] ×100

Where Ac is the absorbance of the control and At is the absorbance of test sample.



MTT assay

1. Prepare cells and test compounds in 96-well plates containing a final volume of 100 µl/well.
2. Incubate for desired period of exposure.
3. Add 10 µl MTT Solution per well to achieve a final concentration of 0.45 mg/ml.
4. Incubate 1 to 4 hours at 37°C.
5. Add 100 µl Solubilization solution to each well to dissolve formazan crystals.
6. Mix to ensure complete solubilization.
7. Record absorbance at 570 nm.



Applications of Microbial Pigments

Biological Significance

Tentatively, microbial pigments with different colors and shades possess defensive properties to protect the organism from UV irradiation thus protecting and increasing their survivability in their surrounding environmental conditions. Antarctic heterotrophic bacteria can able to withstand cold environments by producing carotenoid pigmentation. Some symbiotic or epibiotic pigmented bacteria play a defensive role against the pathogenic and many other predatory organisms, protecting their host organisms. Carotenoid pigments can efficiently scavenge toxic radicals like reactive oxygen species (ROS), reactive nitrogen species (RNS), and some other nonbiological radicals. Several diverse toxic drugs are neutralized by the bacterial melanins and protect the cells from stressful conditions like hyperosmotic stress, high temperature, and starvation like Vibrio cholerae. Some researchers are published revealing marine-origin pigmented bacteria show higher resistance towards heavy metals and antibiotics than non-pigmented bacteria. Neutralization of the compounds containing polyphenols piled in the senescing nodules occurs by the melanin pigments of Rhizobium species. Fungal melanins stabilize the cells from harmful UV radiation. Endophytic fungi protect themselves from insects and other organisms by producing anthraquinones. Diverse organisms produce a natural defensive compound against predators called Tambjamines. Photosynthetic bacteria possess bacteriochlorophylls, proteorhodopsin, and bacteriorhodopsin similar to chlorophyll of the plants. Bacteriorhodopsin present in the bacteria helps them in obtaining energy from a low amount of organic matter. In short, microbial pigments displays many functions like antioxidant properties, UV absorption, virulence, protection from radiation, antibiotic activities, and membrane stabilization. For taxonomic identification and differentiation of various microbes, pigment traits are often used as a biological marker.

Anticancer Potential of Pigments

Around the globe, approximately six million people are suffering from cancer each year. Though many drugs for this prominent disease are being used and some are also in the trial phase, the serious side effects of those drugs have also been reported. Apoptosis is one of the regulated processes for killing tumors without any side effects and is presently focused as a recent cancer treatment option. Various natural products can be obtained from marine source. In previous years nearly about 3000 compounds have been screened from marine sources and some of them also trialed in the clinical phase. Millions of species producing diverse novel agents with therapeutic properties are found in the ocean.

Antimicrobial Activities

Pigments exhibit antimicrobial activities against both Gram-positive and Gram-negative groups of bacteria. *Chromobacteriumviolaceum* produces a violet pigment having an antibacterial effect against *S. typhi* and *S. aurous*. A very strong antimicrobial activity was reported to be shown against *S. aureus, E. coli, S. typhi,* and *K. pneumonia* by a red-colored pigment isolated from *H. alkaliphilus* MSRD1. A study reported the antimicrobial effect of nine different isolated pigments against both Gram-positive and Gram-negative groups of bacteria and concluded that the pigments manifested higher efficacy against Gram-negative pathogenic bacteria than Gram-positive. Several bacterial and fungal species biosynthesize phenazine compounds, anthraquinones, quinones exhibiting a broad range of antimicrobial activities.

Antifungal Activity

Prodiginines like undecylprodigiosin, cycloprodigiosin, and prodigiosin exert antifungal activity against fungi like *Histoplasma, Trichophyton, Asperigillus, Didymella, Cryptococcus, Sacharomyces, Penicillum,* and *Verticillum. Pseudoaltermonas* tunicate produces a yellow pigment called tambjamines possessing antifungal properties. Violacein isolated from *Janthinobacteriumlividum* shows antifungal activity against white root rot causing phytopathogens *Rosellinianecatrix*.

BACTERIA ENUMERATION & CHARACTERIZATION

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Definition

Enumeration of bacteria is defined as the process of determining the number of bacteria in a given sample.

Categories of Bacteria Enumeration

Bacteria enumeration is divided into four categories:

1. Direct Cell Count:

This process counts the number of bacteria in a liquid media or colonies on a plate. In this process, counting chambers is used to directly count the bacterial numbers under the microscope.

2. Indirect Cell Count:

It's the process of estimation of bacterial numbers. This process doesn't involve direct counting of bacterial cell numbers, however, bacterial colonies are calculated through which the concentration of bacterial cells is estimated in the given sample. It's done by the plate count method.

3. Viable Cell Count:

It's counting bacterial cells that are metabolically active and actively dividing and growing in the culture medium.

4. Total Cell Count:

Involves counting the total bacterial cell numbers, including both metabolically active and metabolically inactive/dead microbes.

These four categories of estimating bacteria population are combined in four ways based on the purpose of experimentation in labs. It includes:

- **Direct/Viable:** It's a standard plate count method in which repeated dilutions of a sample determine the number of viable bacteria cells in the given sample. It's used while calculating the number of bacteria in cellular vaccines or milk.
- **Indirect/Viable:** This is a statistical inference about the microbial count based on growth patterns. It's taken into consideration while enumerating the bacterial concentration in soil, laboratory cultures, or water.
- **Direct/Total:** Here, dyes and fluorescent stains are used to make the bacterial cells visible using a fluorescent microscope to aid in counting the bacteria population in the given sample.[2] It's utilized while counting the numbers of bacteria in aquatic samples.

- **Indirect/Total:** Spectroscopy is considered as indirect/total enumeration. With a spectrophotometer, the amount of light passed through the culture is used to estimate the number of microbes present. It's used to estimate the concentration of bacteria in food samples.

Techniques for Bacteria Enumeration

The determination of bacterial cell numbers can be achieved by many methods, including culture turbidity, dry weight of cells, and direct microscopic count. However, among these, the direct viable count technique is the one that's commonly used.

Below are some frequently used bacteria enumeration techniques, their principles, and their differences.

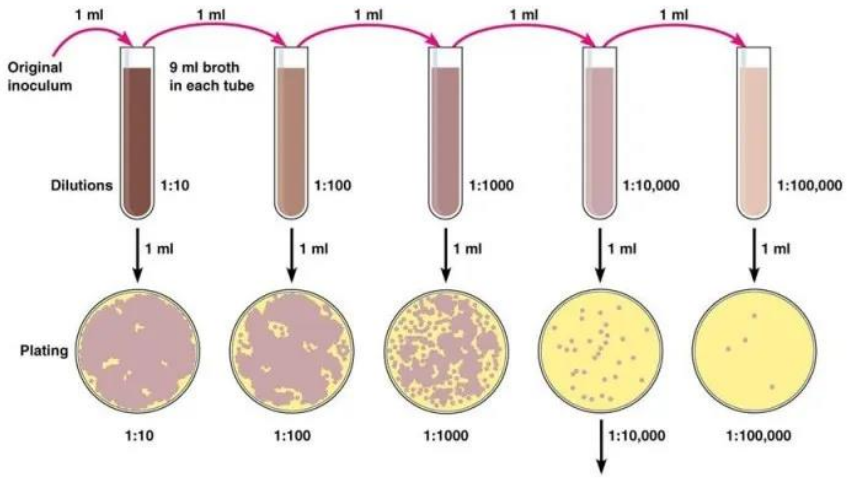
1. Standard Plate Count (Viable Counts)

Bacteria can be present in thousands or millions in a sample, making it difficult to count their numbers. However, serially diluting the cultures makes it easier to determine the count.

After serial dilution, the aliquots of the diluted sample are plated on an appropriate culture media. Then, the plates are incubated, after which the number of colonies formed is counted. This technique is also known as plate count or colony counts.

The techniques that assist in standard plate count include the streak plate and pour plate methods. It's essential to ensure that the plate is not crowded with bacterial colonies because, in such conditions, some cells might either not form colonies or fuse with each other resulting in erroneous results. Statistically, it is most valid to count colonies only on plates with 30 to 300 colonies.

In this method, accurate determination of total cell numbers is only possible if each colony is formed of a single cell. However, it's difficult for one to ensure such a case; that's why the total numbers of cells reported using this method are termed Colony Forming Units (CFUs) rather than cell numbers.



Calculation: Number of colonies on plate × reciprocal of dilution of sample = number of bacteria/ml
(For example, if 32 colonies are on a plate of $^{1}/_{10,000}$ dilution, then the count is 32 × 10,000 = 320,000 bacteria/ml in sample.)

It's calculated as:

Figure: An illustration of serial dilutions and plating techniques.

The number of CFUs per ml of sample = The number of colonies (30-300 plate) X The dilution factor of the plate counted.

2. Turbidimetric Measurement

Turbidity measures the loss of intensity of transmitted light due to the scattering effect of suspended particles.It's simply the amount of cloudiness or turbidity in the sample. It can be caused by silt, sand, mud bacteria, and other microbes or chemical precipitates.

The turbidimetric method is a quick and efficient method to measure and estimate the number of bacteria in a given sample. The method is most preferable when large numbers of cultures are required to be counted.

When bacteria are mixed in a liquid medium, it creates a colloidal suspension that blocks and reflects light as it passes through the culture. The light absorbed by bacterial suspension will estimate the concentration of bacteria in the given sample.

You must note that "*the light absorbed by the culture is directly proportional to the cells' concentration.*"

Even though the turbidity measurement technique is faster than the standard plate count, it is initially required to match the measurement values with cell numbers, which requires lab personnel to combine the standard plate count method with the turbidity technique. The procedure followed include:

- First, the turbidity of different concentrations of a given bacterial species in a culture medium is determined.
- Using standard plate count, the number of viable microorganisms per milliliter of the samples is estimated.
- A standard curve is drawn, with turbidity or optical density readings corresponding to specific numbers of living organisms.

Once drawn, the standard graph can be directly used for subsequent turbidity measurement and calculating the number of viable bacterial cells, eliminating the process of time-consuming standard plate count.

The method utilizes instruments such as a spectrophotometer or colorimeter to measure the turbidity in the given sample. They contain a light source and a light detector separated from the sample compartment.

The cultures are poured into a cuvette and kept in the instrument. When light passes through the culture sample, cells interfere with them, which provides an output value on the machine that helps to calculate the bacteria numbers in the cultures.

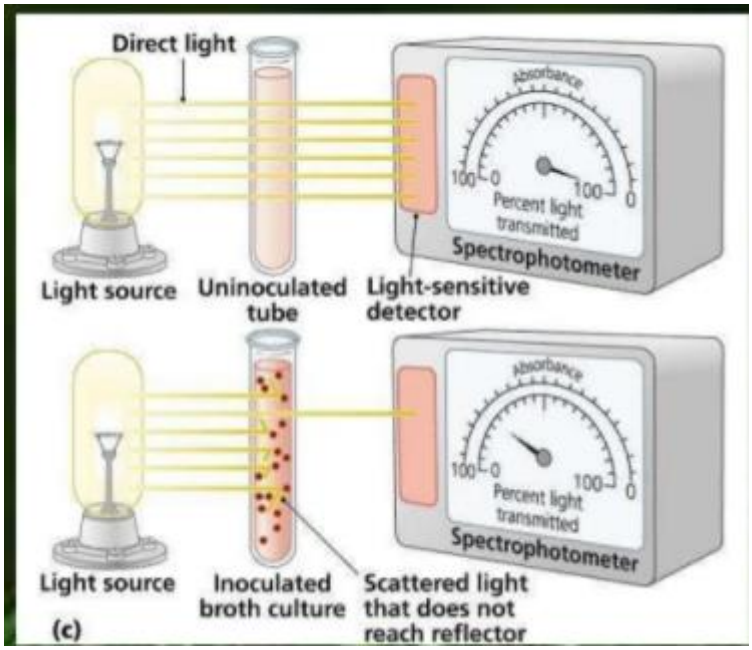


Figure: An illustration of turbidity measurement using a spectrophotometer.

Before experimenting, it's essential to adjust the spectrophotometer at 100% transmittance (which means 0% absorbance), which is done using a sample of the uninoculated medium.

At last, the percentage of transmittance of different bacterial culture dilutions is measured, and the obtained values are converted to optical density using the formula:

Absorbance (O.D.) = 2 – log % Transmittance

The wavelength is determined based on the color of the solution. For example, if it's white, 420 nm is used, 540 nm if the color is yellow, and 600-625 nm if the solution is in the range of yellow to brown color.

3. Direct Microscopic Count

The direct microscopic count is used for quantitative enumeration of bacteria in water, food, milk, and air samples.

It is done by spreading a measured volume of the sample over a predetermined area of a slide, counting representative microscopic fields, and transforming the average values into appropriate volume-area factors.

The counting chambers used for the procedures include Petroff-Hauser and Levy counting chambers. The Petroff-Hauser is a thick microscope slide with a chamber in the middle of 0.02 mm (1/50 mm) deep, and it also has improved Neubauer rulings and an etched grid in the chamber. The ruling is the centerline of a group of three squares in which cells are counted.

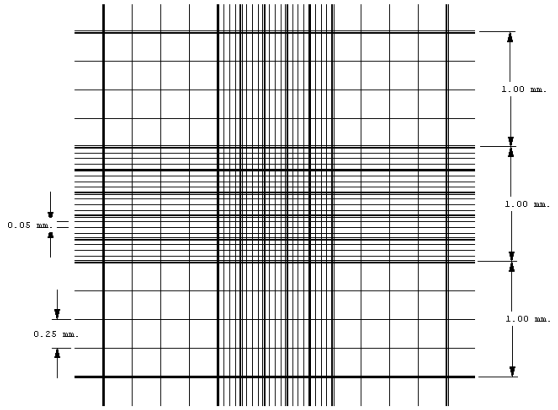


Figure: An illustration of Petroff-Hauser counting chamber.

A single drop of culture is applied in this counting method using a Pasteur pipette. The cell numbers in the given sample are counted directly in 10-20 high microscope fields. Based on the average number of cells per field, the number of bacteria per milliliter of the original sample can be obtained.

The final concentration of cells in the given sample is calculated using the formula:

Total cells counted x 2.0×10 7 x dilution factor/ # small squares counted = cells/ml

Using fluorescent dyes

The direct microscopic count is also done using fluorescent dyes. These dyes are popular in labs because of their ability to stain all bacterial species in a given sample, a particular species, or a specific component of cells. A few examples of fluorescent dyes are cyanoditolyl tetrazolium chloride (CTC), auramine, acridine orange, and rhodamine.

The most common fluorescent dye used to stain bacteria is acridine orange. In this method, a known sample volume is stained with acridine orange and then filtered through a 0.22 μm filter. The filter traps the bacteria that are then examined under the fluorescent microscope. By counting bacteria in a defined area of the filter, the concentration of bacteria in the original sample can be determined.

Conclusion

Bacteria enumeration is the process of determining the number of bacterial cells in a given sample. The counting of bacterial cells has four categories based on the purpose of the experiment: direct, indirect, viable, and total cell count. The categories are combined in four different ways to serve the experimental purpose in different techniques.

Many methods have been developed to count the numbers of bacteria in labs, but the most frequently used ones are standard plate count, turbidimetric method, and direct microscopic count.

The techniques are essential in food and beverage industries, where counting the numbers of bacteria in the given food or beverage sample is essential to learn if they are safe to consume and are not contaminated. Besides this, bacteria enumeration also has applications in agriculture and processing industries.

Despite making the enumeration process easier and smoother, the available techniques have several limitations. Thus, offering young researchers the chance to develop techniques that cover the limitations and provide a faster approach for the enumeration process.

BIOCHEMICAL TESTS FOR IDENTIFICATION OF BACTERIA

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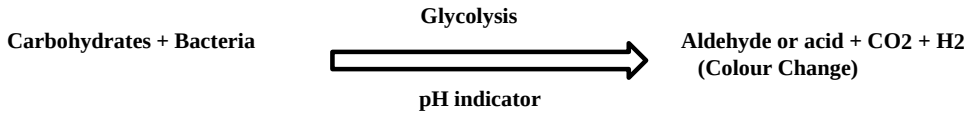
1) Carbohydrate Fermentation

Aim

To determine the ability of microorganisms to degrade and ferment carbohydrate with the production of acid and gas

Principle

Most microorganisms use carbohydrate differently depending on their enzyme's components. In fermentation, substrate and alcohols undergo anaerobic dissimilation and produce an organic acid (For example lactic acid, formic acid or acetic acid). The pH indicator Phenol Red is used to detect the production of acid, which is red at a neutral pH 7 and changes to yellow at a slightly acidic pH of 6.8. This indicates a positive reaction.



In some cases, acid production is accompanied by the evaluation of gas such as Hydrogen or Carbon dioxide. To detect the presence of gas produced or Durham's tube (an inverted inner vial) is placed in the fermentation broth, in which the evaluation of gas will be visible as a bubble.

Cultures that are not capable of fermenting any carbohydrate and not producing concomitant evolution of gas are noted. This is a negative reaction.

Materials Required

8 ml Test Tube, Durham's Tube, Phenol Red Indicator, Sugar (Glucose, Lactose, Sucrose)

Procedure

•Using sterile technique, culture was inoculated into its appropriately labeled medium by means of loop inoculation.

•Care was taken during this step not to shake the fermentation tube.

•1 tube of each fermentation broth was kept uninoculated as a comparative control.

•All the tubes were incubated at 37°C for 24 hours and the reaction was observed.

Observation

All carbohydrate broth cultures were observed for colour and presence or absence of gas bubble by comparing with the uninoculated tube (control).

Table-Carbohydrate–FermentationTest		
Glucose	Lactose	Sucrose
Fermentedwithacidproduction only eg.*S.aureus*	Fermented with acid production only Eg:*S. aureus*	Fermentedwithacidproduction only Eg:*S.aureus*
Fermentedwithacidandgasproductioneg.*E.coli,Klebsiella*	Fermentedwithacidandgasproduction Eg:*E. coli, Klebsiella*	Fermentedwithacidand gas productionEg: *E. coli, Klebsiella*
Non-Fermentingeg.*Acinoetobacter*	Non- FermentingEg:*S. typhi* *S.paratyphi* *Pseudomonassp.*	Non-fermentingEg:*S. typhi* *S.paratyphi* *Pseudomonassp.*

2) Oxidation – Fermentation Test

Aim

To determine the oxidation fermentation characteristics of microorganisms

Principle

This method depends upon the use of semisolid tube medium containing the carbohydrate (Glucose) together with a pH indicator. The acid is produced only at the surface of medium where conditions are aerobic the attack on the medium where conditions are aerobic the attack on the sugar is oxidative. If acid is produced throughout the medium including lower layers and where the conditions are aerobic breakdown is fermentative.

Fermenting organism (*Enterobacteriaceace, Vibrio*) produce an acidic reaction throughout the medium in the covered (anaerobic) as well as open (aerobic) tube. Oxidizing organisms (*Pseudomonas*) produce an acidic reaction only in the open tube. Organisms that cannot breakdown carbohydrate aerobically/anaerobically (alkali genes faecalis) produce an alkaline reaction in the open tube and no change in the covered tube. This medium may be used for detecting gas production and motility.

Materials Required

Bacterial broth culture, D-F medium, liquid paraffinaol

Procedure:

•Using sterile technique, two tubes of medium were inoculated by stabbing with sterile urine.

•Two inoculated tubes were used as control.

•Liquid paraffin was poured over the medium to form a layer about 1cm in depth into one of the tube of each pair.

•The tubes were incubated at 37°C for 24-48hrs was observed.

Observation

The tubes were observed for the colour of the medium and the type of metabolism was recorded.

Table6.Oxidation –FermentationTest	
OXIDATIVE	FERMENTATIVE
Eg.*Pseudomonas*	Eg.*Klebsiella* *S. typhi* *S.paratyphi A* *E.coli*

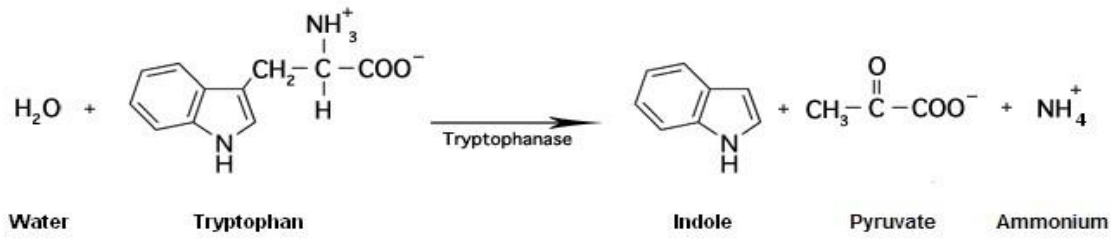
3) Indole Production Test

Aim

To determine the ability of microorganisms to decompose the amino acid tryptophan to indole

Principle

Tryptophan an essential amino acid oxidized by some bacteria by the enzyme tryptophanase resulting in the formation of indole, pyruvic acid and ammonia. In this experiment, the medium contains the substrate tryptophan which is utilized by the microorganisms.



Enzymatic Degradation of Tryptophan

This ability to hydrolyse tryptophan with the production of indole is not a characteristic of all microorganisms and therefore serves as a biochemical mask. The presence of indole is detected by adding Kovac's reagent, which produces a cherry red reagent layers. This colour is produced by the reagent which is composed of Paradimethylaminobenzaldehyde yielding the cherry red colour

Indole Reaction with Kovac's Reagent

Culture producing a red reagent layers following addition of the Kovac's reagent are indole positive. The absence of red colouration demonstrates that the substrate tryptophan was not hydrolyzed and indicating indole negative reaction.

Another reagent used is Ehrlisch's reagent. It's believed to be more sensitive than Kovac's reagent and is recommend for the detection of indole production by anaerobic and non-fermentative Gram-

negative organism Kovac's reagent was used usually initially to classify the members of *Enterobacteriaceace*family.

Materials Required

15 ml test tubes, bacterial culture, peptone water, Kovac's reagent

Procedure

•The peptone water tubes were inoculated with bacterial broth culture using sterile needle technique.

•An uninoculated tube was kept as control.

•Both tubes were incubated at 37°C for 24-48 hours.

•After proper incubation, 1 ml of Kovac's reagent was added to both tubes including the control.

•The tubes were shaken gently after an interval for 10 – 15 minutes.

Observation

The tubes were observed for the colour in the top reagent layer.

Note

Development of cherry red colour in the top layer of the tube is a positive test. Absence of red colouration is indole negative.

Examples

Positive: *E. coli, Proteus vulgaris*

Negative: *Klebsiella sp., Proteus mirabilis*

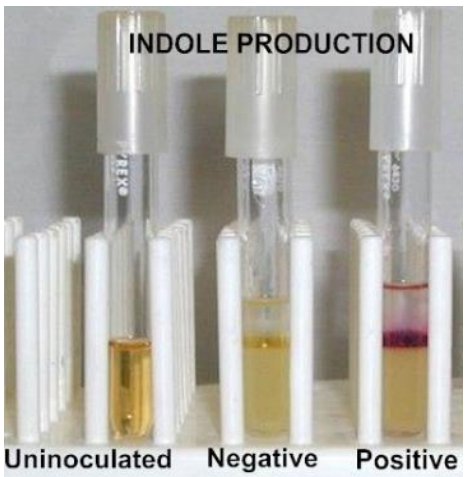


Fig.Indole Production Test

4) Methyl Red Test

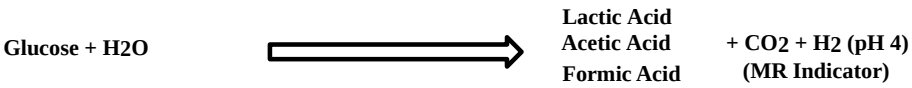
Aim

To determine the ability of microorganism to oxidize glucose with the production and stabilization of high concentrations of acid end products

Principle

All enteric organisms oxidize glucose for energy production and the end products of this process will vary depending on the specific enzymatic pathway present in the bacteria. In this test, the pH indicator methyl red detects the presence of large concentrations of acidic red detects the presence of large concentrations of acidic products. The test can be used in differentiating *Escherichia coli* and *Enterobacter aerogenes* (both coliform bacteria) that are used as indicator of the sanitary quality of water, foods etc.

Both of these organisms initially produce organic acid end productsduring the early incubation period. The low acid end products produce acidic pH (4) which is stabilized and maintained by *E. coli* at the end of incubation. During the later incubation period *Enterobacter aerogenes* enzymatically converts these acids into nonacid end products such as 2,3 butanedial and acetyl methyl carbinol (pH 6).



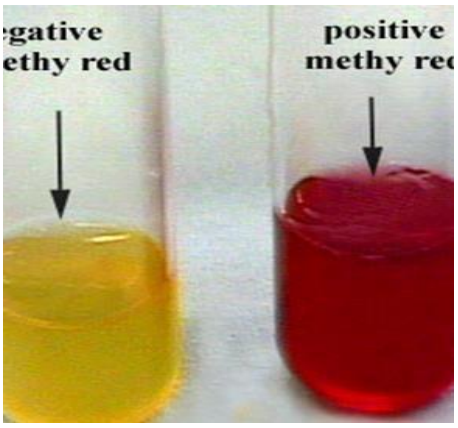


Fig. 16. Methyl Red Test

At a pH of 4, Methyl red indicator will turn red throughout the tube, which is indicating of a positive test. At pH 6, still indicating the presence of acid but with a lower hydrogen ion concentration, the indicators turn Yellow, which is indicating the negative test.

Materials Required

MR broth, 24 hours broth cultures, Methyl red indicator, inoculating loop

Procedure

•Using sterile technique experimental organisms were inoculated into appropriately labeled tubes containing MR broth by means of loop inoculation.

•Uninoculated tube was kept as control

•Both tubes were incubated at 37°C for 24-48 hours.

•After proper incubation 5 drops of MR indicator was added to both tubes including control.

•It was mixed well and colour was observed.

Observation

The tubes were observed for changes in the colour of Methyl Red.

Interpretation

The colour of MR reagents remaining red is a positive test and the colour turning to yellow is negative.

Examples

MR positive – E. coli, Proteus sp; Salmonella sp. MR negative – Klebsiella, Enterobacter sp.

5) Voges – Proskauer Test

Aim

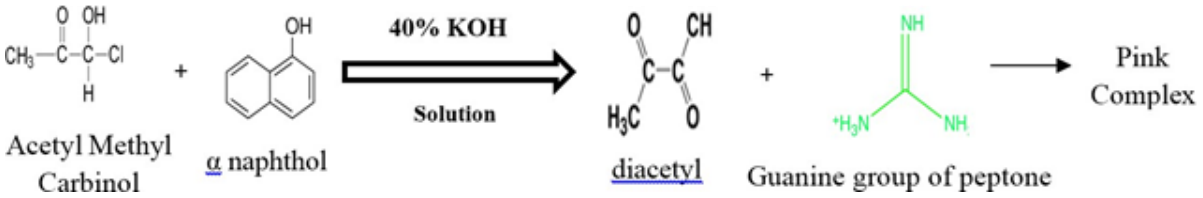
To determine the ability of many microorganisms to produce acetone (acetyl methyl carbinol) during fermentation of glucose

Principle

This determines the ability of many bacteria to ferment carbohydrates with the production of non-acidic / neutral end products, acetyl methyl carbinol or its reduction product, acetyl methyl carbinol or its reduction products, acetyl- methyl carbinol or its reduction product 2,3 Butylene glycol from the organic acids.

The reagent used in this test, Barrett's reagent, consists of a mixture of alcoholic α- naphthol and 40% potassium hydroxide solution. Detection of the acetyl methyl carbinol requires this end product to be oxidized to a diacetyl compound. This reaction will occur in the presence of α- naphthol catalyst and a guanidine group that is present in the peptone. At a result, a pink complex a guanidine group that is present in the peptone. As a result, a pink complex is complex is formed imparting a rose colour to the medium.

Acetyl Methyl Carbinol reaction with Barrett's reagent



Development of deep rose colour in culture with in a minute following the addition of Barrett's reagent is indicative of presence of the acetyl methyl carbinol and represents a positive result. The absence of rose colouration is a negative result.

Procedure

•Using sterile technique, the experimental organism was inoculated into VP broth by means of loop inoculation.

•One tube is kept uninoculated as control.

•The tube will be incubated at 37°C for 24-48 hours.

•After proper incubation, about 3 ml of Barrett's reagent A & 1 ml of Barrett's reagent B was added into both tubes including control.

•The tubes were shaken gently for 30 seconds with the caps off to expose the media to oxygen.

•The reaction was allowed to complete in 15 – 30 minutes and tubes were observed.

Observation

The tubes were observed for the development of crimson red colour.

Note: the colour may be more intense at the surface.

Interpretation

Red colour formation indicates a positive test and colour change is negative. eg. Positive – *Klebsiella sp., Enterobacter*

Negative – *E. coli, Proteus sp.*

6) Citrate Utilization Test

Aim

To determine the ability of a microorganism to utilize citrate as the sole source of carbon and as energy source for the growth and ammonium salt as a sole source of nitrogen

Principle

Citrate test is used to differentiate among enteric bacteria on the basis of their ability to utilize / ferment citrate as the sole carbon source. In the absence of glucose or lactose some microorganisms utilize citrate as a carbon source. This ability depends on the presence of citrase enzyme that facilitates the transport of citrate in the cell. Citrate, the first major intermediate in Krebs's cycle is produced by the condensation of active acetyl CoA with oxalo acetic acid and acetate. These products are then enzymatically converted to pyruvic acid and carbon dioxide. During this reaction themedium becomes alkaline; CO_2 combines with sodium and water to form carbonate, an alkaline product. This changes the bromothymol blue indicator in the medium from green to Prussian blue.

Citrate test is preferred / performed by inoculating the microorganisms in to an organic synthetic medium. Simmons citrate agar (solid) or Koser's citrate medium (liquid) in which sodium citrate is the only source of carbon and energy.

Bromothymol blue is green when acidic (pH 6.8 and below). When alkaline (pH 7.6 and above). Formation of blue colour constitutes a positive test. Citrate negative culture will show no growth and the medium will remain green.

Materials Required

Bacterial broth, Simmons Citrate Agar Slants, Inoculation Loop

Procedure

•Using sterile technique Simmons citrate agar slant was inoculated with the test organism by means of a stab and streak inoculation.

•An uninoculated tube was kept as control.

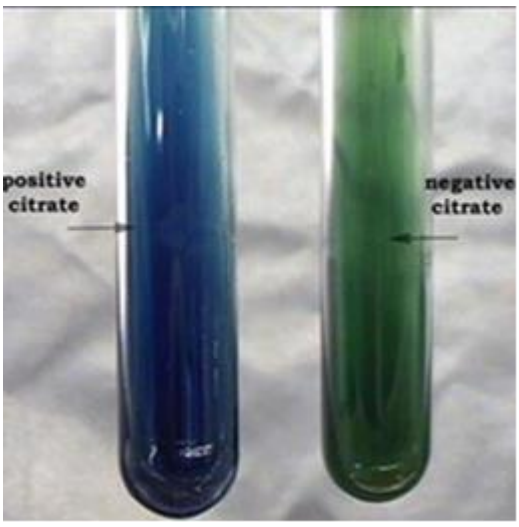
•Both tubes were incubated at 37°C for 24 – 48 hours & was observed

Observation

The tubes were observed for growth and colouration of the medium.

Interpretation

Colour of the medium if turned blue, a positive result is indicated. Colour of the medium remains as green, indicates a negative result.



7) Nitrate Reduction Test

Aim

To determine the ability of bacteria to produce an enzyme nitrate reductase

Principle

The reduction of nitrate by some aerobic and facultative anaerobic microorganisms occurs in the absence of molecular oxygen an anaerobic process whereby the cell uses in organic substances such as nitrates or sulphates to supply oxygen that is subsequently utilized as a final hydrogen acceptor during energy formation. The biochemical transformation may be utilized as follows:

$$NO_3^- + 2H^+ + 2e^- \xrightarrow{\text{Nitrate reductase}} NO_2 + H_2O$$

Some organisms possess the enzymatic capacity to act further on nitrates to reduce them to ammonia or molecular nitrogen. These reactions may be described as follows:

$$NO_2^- \longrightarrow NH_3^+$$

Nitrate reduction can be determined by cultivating organisms a nitrate broth medium. The medium is basically a nutrient broth supplemented with 0.1% potassium nitrate (KNO3) as the nitrate substrate. In addition, the medium is made into a semisolid by the additional of 0.1% agar. The semisolid impedes the diffusion of oxygen in to the medium, there by favoring the anaerobic requirement necessary for nitrate reduction. An organism's ability to reduce nitrate to nitrite is determined by the addition of two reagent solution A, which is sulphanlic acid followed by solution B, which is α-napthylamine followed reduction, the addition of solution A and B will produce an immediate cherry red colour.

$$NO3^- \xrightarrow{\text{Nitrate Reductase}} NO2^-$$

Cultures not producing a colour change suggest one of two possibilities

•Nitrates were not reduced by the organism

•The organism possessed such potent nitrate reductase enzymes that nitrate were rapidly reduced beyond nitrates to ammonia or even molecular nitrogen.

This test determines the production of an enzyme called nitrate reductase, resulting in the reduction of nitrate (NO3). With this enzyme, nitrate is reduced to nitrite (NO2). It then forms nitrous acid that reacts with the first reagent sulphanlic acid, and that reacts with the other reagent α-napthylamine to form a red colour. The development of red colour, therefore, verifies that nitrates were not reduced to nitrites by the organism. If nitrites were reduced a negative nitrate reduction had occurred. If the addition of zinc does not produce colour change, the nitrates in the medium were reduced beyond

nitrites to ammonia or nitrogen gas. This is a positive reaction or result. Reduction of nitrate is generally an anaerobic respiration in which an organism derives its oxygen from nitrate.

Materials Required

Bacterial broth, Nitrate broth, Nitrate reagent and inoculation loop.

Procedure

•Using sterile technique the test organism was inoculated in to nitrate broth by means of loop inoculation.

•An uninoculated broth was kept as control.

•Both tubes were incubated at 37°C for 24-48 hours.

•After proper incubation equal amounts of nitrate reagent (solution A & B) were added to nitrate broth Cultures and to the control tube and the reaction was observed

Observation

The tubes were observed to see a red colour has been developed or not.

A minute quantity of zinc was added to cultures in which no red colour was developed and it was observed to see if red colour has been developed or not.

Interpretation

Development of red colour indicates nitrate positive and no colour change indicates a negative test.

Eg: Positive: all members of *Enterobacteriaceace*

Negative:*Haemophilusduceryi.*

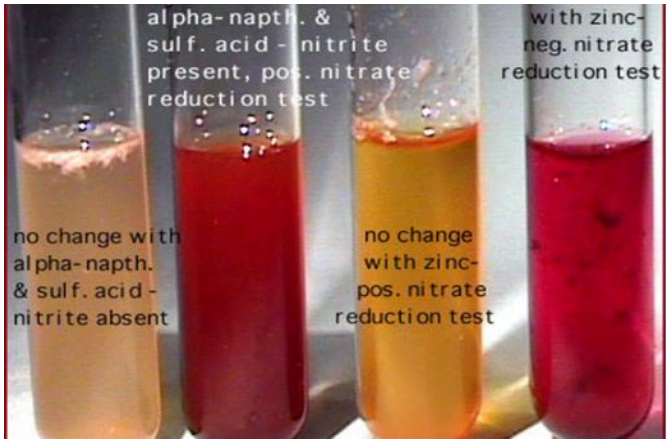


Fig. 19. Nitrate Reduction Test

8) Urease Test

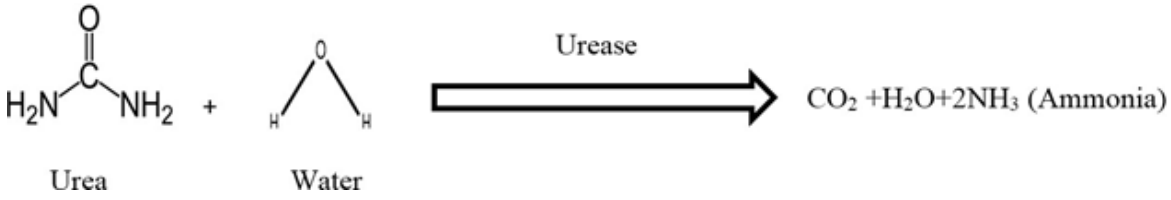
Aim

To determine the ability of microorganism to degrade urea by means of the enzyme urease

Principle

Urease is a hydrolytic enzyme that attacks nitrogen and carbon bond in amide compounds such as urea and forms the alkaline end products ammonia. The presence of urease is detectable when the organisms are grown in a urea broth medium containing the pH indicator phenol red. As the substrate

urea is split into its products, the phenol red to turn to a deep pink. This is a positive reaction for the presence of urease. Failure of deep pink colour to develop is evidence of negative reaction



Procedure

•Using sterile technique, the test organism was inoculated the media by means of loop of inoculation.

•An uninoculated tube was kept as control.

•The tubes were incubated at 37°C for 24-24 hours and the reaction was observed.

Observation

The tubes were observed to see if pink colour has developed or not

Interpretation

Development of pink colour indicator a positive test and no colour change shows a negative test, Eg

Urease Positive – *Klebsiella sp., Proteus sp.*

Urease Negative – *E. coli, Salmonella sp.*

9) Mannitol Motility Test

Aim

To detect whether the given organism is motile and also mannitol is fermenting or not

Principle

Mannitol motility test medium is an example of semisolid agar media; motile bacteria swarm and give a diffused spreading growth that is easily recognized by the naked eye. The final sterile medium should be quite clear and transparent. After incubating the stabbed culture, non-motile bacteria generally give growth that are confined to stab line and have sharply defined margins leaving the surrounding medium clearly transparent. Motile bacteria typically give diffused, hazy growth that spreads throughout the medium rendering it slightly opaque. This test also helps to identify whether the microorganisms ferment Mannitol or not. It produces acidic end products which in turn change the red colour of phenol red indicator to yellow.

Materials Required

Bacterial culture broth, mannitol fermentation media (semisolid) and inoculation loop

Procedure

•Using sterile technique, the test organism was inoculated in to the medium using stab inoculation method.

•An uninoculated tube was kept as control.

•Both tubes were incubated at 37°C for 24-48 hours and the reaction was observed.

Observation

The tubes were observed for motility and also for colour changes from red to yellow.

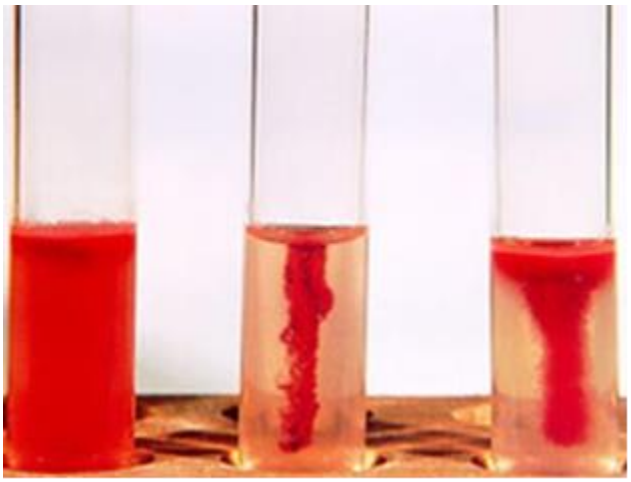
Interpretation

Diffused growth– Motile bacteria eg:*Pseudomonas sp.*

Growth at stab line only – non-motile bacteria eg: *Staphylococcus aureus* only

Red colour – Mannitol non- fermenting eg: *Bacillus cereus*

Yellow colour - Mannitol fermenting eg*: E. coli*



10) Triple Sugar Iron Agar Test

Aim

To identify the microorganisms based on the ability to ferment the carbohydrates (Glucose, Sucrose and Lactose)

Principle

The triple sugar- iron agar test is designed to differentiate among the different groups or genera of the *Enterobacteriaceace*, which are all Gram negative bacilli capable of fermenting glucose with the production of acid and to distinguish them from other gram negative intestinal bacilli. This differentiation is based on the differences in carbohydrate fermentation patterns and hydrogen sulfide production by the various groups of intestinal organisms. Carbohydrate fermentation is indicated by the presence of gas and a visible colour change of the pH indicator, phenol red. The production of hydrogen sulphide in the medium is indicated by the formation of a black precipitate that will blacken the medium in the butt of the tube.

To facilitate the observation of carbohydrate utilization patterns, TSI Agar contains three fermentative sugars, lactose and sucrose in 1% concentrations and glucose in 0.1% concentration. Due to the production of acid during fermentation, the pH falls. The acid base indicator Phenol red is incorporated for detecting carbohydrate fermentation that is indicated by the change in colour of the

carbohydrate medium from orange red to yellow in the presence ofacids. In case of oxidative decarboxylation of peptone, alkaline products are produced and the pH rises. This is indicated by the change in colour of the medium from orange red to deep red. Sodium thiosulfate and ferrous ammonium sulfate present in the medium detects the production of hydrogen sulfide and is indicated by the black colour in the butt of the tube.

Carbohydrate fermentation is indicated by the production of gas and a change in the colour of the pH indicator from red to yellow. To facilitate the detection of organisms that only ferment glucose, the glucose concentration is one-tenth the concentration of lactose or sucrose. The amount of acid production in the slant of the tube during glucose fermentationoxidizes rapidly, causing the medium to remain orange red or revert to an alkaline pH. In contrast, the acid reaction (yellow) is maintained in the butt of the tube since it is under lower oxygen tension.

After depletion of the limited glucose, organisms able to do so will begin to utilize the lactose or sucrose. To enhance the alkaline condition of the slant, free exchange of air must be permitted by closing the tube cap loosely. If the tube is tightly closed, an acid reaction (caused solely by glucose fermentation) will also involve the slant.

Materials Required

Bacterial broth cultures, TSI agar slants, Inoculation Loop.

Procedure

•Using sterile technique, the test organism was inoculated into the media by means of stab and streak inoculation.

•An uninoculated tube was kept as control

•Both tubes were incubated at 37°C for 24 hours and the reaction was observed

Observation

The tubes were observed for the colour of both the butt and slant and also gas production by means of cracks or bubble or blackness of butt.

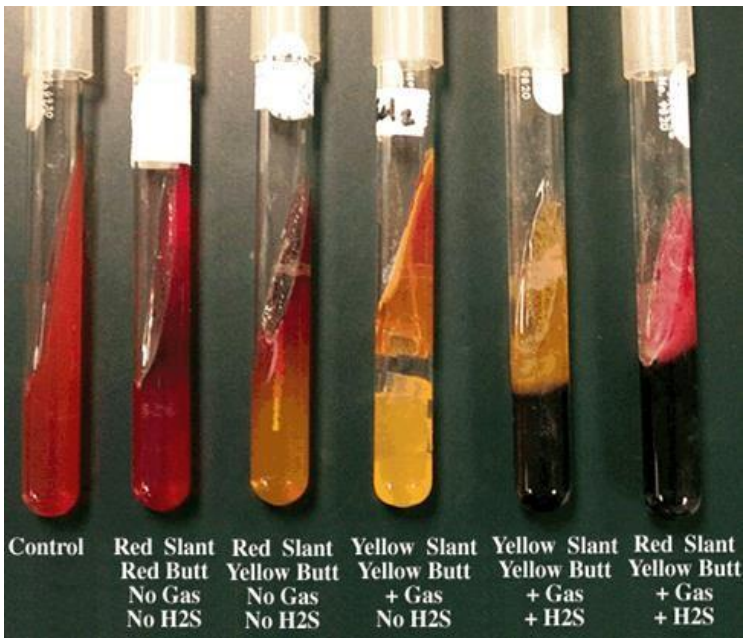


Fig. .Triple Sugar Iron Agar Test

Interpretation

•A/A: ferments glucose and either sucrose, lactose, or both.

•K/A: does not ferment lactose or sucrose; does ferment glucose.

•K/K: a non-fermenter.

•Black precipitate in stab: produces H2S (and ferments glucose).

Observation	Interference	Examples
A/A without gas and H_2Sproduction	AcidSlant/ Acid butt without gas & H_2Sproduction	*Staphylococcusaureus*
A/A with gas and withoutH_2Sproduction	AcidSlant/ Acidbutt with gas & without H_2Sproduction	*E.coli,Klebsiella*
K/A with gas and withoutH_2Sproduction	Alkalineslant/ Acidbutt with gas & without H_2Sproduction	*Salmonellaparatyphi*A
K/K without gas and H_2Sproduction	Alkalineslant/ Acidbutt without gas & H_2Sproduction	*Pseudomonas* sp.
K/AwithH2Sproduction	Alkaline slant / Acid buttwithH2Sproduction	*Salmonellatyphi*

11) Catalase Test

Aim

To demonstrate the presence of catalase in an organism.

Principle

Catalase is an enzyme, which is produced by microorganisms that live in oxygenated environments toneutralize toxic forms of oxygen metabolites and H2O2. The catalase enzyme neutralizes the bactericidal effects of hydrogen peroxide and protects them. Anaerobes generally lack the catalase enzyme.

Catalase mediates the breakdown of hydrogen peroxide H_2O_2 into oxygen and water. To find out if a particular bacterial isolate is able to produce catalase enzyme, small inoculums of bacterial isolate is mixed into hydrogen peroxide solution (3%) and the rapid elaboration of oxygen bubbles occurs. The lack of catalase is evident by a lack of or weak bubble production.

Catalase-positive bacteria include strict aerobes as well as facultative anaerobes. They all have the ability to respire using oxygen as a terminal electron acceptor.

Catalase-negative bacteria may be anaerobes, or they may be facultative anaerobes that only ferment and do not respire using oxygen as a terminal electron acceptor (ie. Streptococci).

Uses

•The catalase test is primarily used to distinguish among Gram-positive cocci: Member of the genus *Staphylococcus* is catalase-positive, and members of the genera Streptococcus and Enterococcus are catalase-negative.

•Catalase test is used to differentiate aero tolerant strains of Clostridium, which are catalase negative, from Bacillus species, which are positive.

•Semi quantitative catalase test is used for the identification of Mycobacterium tuberculosis

•Catalase test can be used as an aid to the identification of Enterobacteriaceace. Members of Enterobacteriaceace family are Catalase positive.

Materials Required

24 hours old bacterial culture, glass slide, petridish, 3% H_2O_2, applicator sticks

Procedure

•Transfer a small amount of bacterial colony to a surface of clean, dry glass slide using a loop or sterile wooden stick

•Place a drop of 3% H_2O_2 on to the slide and mix.

•A positive result is the rapid evolution of oxygen (within 5-10 s) as evidenced by bubbling.

•A negative result is no bubbles or only a few scattered bubbles.

•Dispose of your slide in the biohazard glass disposal container.

Fig. Catalase Test

Precautions

•Do not use a metal loop or needle with H_2O_2; it will give a false positive and degrade the metal.

•If using colonies from a blood agar plate, be very careful not to scrape up any of the blood agar as blood cells are catalase positive and any contaminating agar could give a false positive.

Observation

The release of bubbles was observed and compared with control.

Interpretation

Bubble Formation : Catalase Positive No Bubble Formation: Catalase Negative Examples

Catalase Positive :*Staphylococcus aureus*

Catalase Negative: *Streptococcus pyogenes*

Note

Care must be taken while performing catalase test of growth from blood agar plate because blood (RBC) contains RBC catalase.

12) Oxidase Test

Aim

To test the production of oxidase bacteria

Principle

The oxidase test is a key test to differentiate between the families of *Pseudomonadaceae* (ox +) and *Enterobacteriaceace* (ox-), and is useful for speciation and identification of many other bacteria those that have to use oxygen as the final electron acceptor in aerobic respiration. The enzyme cytochrome oxidase is involved with the reduction of oxygen at the end of the electron transport chain.

There may be different types of oxidase enzymes produced by bacteria. The colorless redox reagent, tetra methyl-p-phenylenediamine dihydrochloride (or dimethyl) used in the test will detect the presence of the enzyme oxidase and reacting with oxygen, turn a colour. The oxidase reagent contains a chromogenic reducing agent, a compound that changes color when it becomes oxidized, so it acts as an artificial electron acceptor for the enzyme oxidase. The oxidized reagent forms the coloured compound indophenol blue.

Materials Required

Oxidase disc, 24 hours old test organism, applicator stick or glass rod.

Procedure

•The test organisms were rubbed over the reagent impregnated, filter paper disc using sterile applicator sticks or glass rod.

•Controls were also kept along with the test and the reaction was observed within 10 seconds.

Observation

The colour changes to purple were observed with the prescribed time.

Important

Acidity inhibits oxidase enzymes activity therefore the oxidase test must not be performed on colonies that produce fermentation on carbohydrates containing media like Mac Conkey Agar.

Interpretation

Formation of purple colour indicates a positive test. No colour changes show a negative test. eg. Oxidase Positive: *Pseudomonas sp., Vibrio sp.*

Oxidase Negative: *E. coli, Klebsiella*

Precautions

•The test reagent is to be freshly prepared

•Nichrome wire is not used to take bacterial growth

•Cultures should not be very cold

•Culture from selective media should not be used

•The colour changes should be observed within the prescribed time

Fig. Oxidase Test

13) Coagulase Test

Aim

To distinguish coagulase producing *Staphylococcus aureus* from other species of Staphylococcus

Principle

Staphylococcus aureus is known to produce coagulase, which can clot plasma into gel in tube or agglutinate cocci in slide. This test is useful in differentiating S. aureus from other coagulase-negative staphylococci. Most strains of *S.aureus* produce two types of coagulase, free coagulase and bound coagulase. While free coagulase is an enzyme that is secreted extracellular, bound coagulase is a cell wall associated protein. Free coagulase is detected in tube coagulase test and bound coagulase is detected in slide coagulase test. Slide coagulase test may be used to screen isolates of *S. aureus* and tube coagulase may be used for confirmation. While there are seven antigenic types of free coagulase, only one antigenic type of bound coagulase exists. Free coagulase is heat labile while bound coagulase is heat stable.

Slide Coagulase Test: The bound coagulase is also known as clumping factor. It cross-links α and β chain of fibrinogen in plasma to form fibrin clot that deposits on the cell wall. As a result, individual coccus sticks to each other and clumping is observed.

Tube Coagulase Test: The free coagulases secreted by *S. aureus* act with coagulase reacting factor (CRF) in plasma to form a complex, which is thrombin. This converts fibrinogen to fibrin resulting in clotting of plasma.

Materials Required

EDTA anticoagulant human plasma, clean glass slide, test tubes, pipettes, distilled water and inoculation loop.

Procedure

Slide Coagulase Test: Dense suspensions of Staphylococci from culture are made on two ends of clean glass slide. One should be labeled as "test" and the other as "control". The control suspension serves to rule out false positivity due to auto agglutination. The test suspension is treated with a drop of citrated plasma and mixed well. Agglutination or clumping of cocci within 5-10 seconds is taken as positive. Some strains of *S. aureus* may not produce bound coagulase, and such strains must be identified by tube coagulase test.

Observation

The slides were observed for clumping or not within prescribed time.

Interpretation

Clumping formation - Positive reaction No clumping formation – Negative reaction Tube Coagulase Test

Three test tubes are taken and labeled "test", "negative control" and "positive control". Each tube is filled

with 0.5 ml of 1 in 10 diluted rabbit plasma. To the tube labeled test, 0.1 ml of overnight broth culture of test bacteria is added. To the tube labeled positive control, 0.1 ml of overnight broth culture of known [*S. aureus* is added and to the tube labeled negative control, 0.1 ml of sterile broth is added. All the tubes are incubated at 37°C and observed up to four hours. Positive result is indicated by gelling of the plasma, which remains in place even after inverting the tube. If the test remains negative until four hours at 37°C, the tube is kept at room temperature for overnight incubation.

Observation

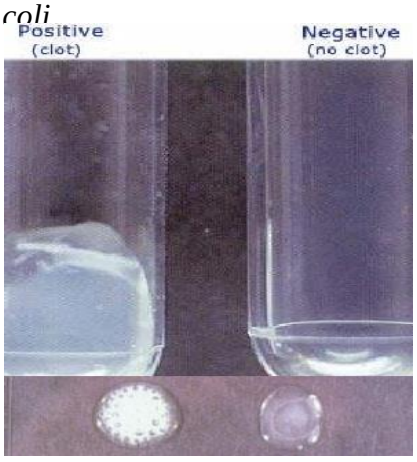
The tubes were observed for clotting in the prescribed time.

Interpretation

Clot formation - Positive reaction No clot formation – Negative reaction Examples

Coagulase Positive: *Staphylococcus aureus*

Coagulase Negative: *E. coli*



ELECTROPHORESIS AND ITS APPLICATION

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Electrophoresis

The term electrophoresis describes the migration of a charged particles under the influence of an electric field. Various essential biological molecules, such as amino acids, peptides, proteins, nucleic acids, nucleotides, have ionizable group, which at given pH exist in a solution as electrically charged species either as cation (+ve) and anion (-ve) are separated by electrophoresis.Under the influence of electric field these charged particles will migrates either to cathode or anode depending on the nature of their net charge

Principle of electrophoresis:

Electrophoresis is based on the phenomenon that most biomolecules exist as electrically-charged particles, possessing ionizable functional groups. Biomolecules in a solution at a given pH will exist as either positively or negatively charged ions. When a potential difference is applied, the molecules with different overall charge will begin to separate owing to their different electrophoretic mobility. Even the molecules with similar charge will begin to separate if they have different molecular sizes, since they will experience different frictional forces. Therefore, some form of electrophoresis rely almost totally on the different charges on the molecules for separation while some other form exploits difference in size (molecular size) of molecules.

Different types of electrophoresis:

1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

2. Immunoelectrophoresis

3. Cellulose acetate electrophoresis

4. Pulse field gel electrophoresis (PAGE)

5. Agarose gel electrophoresis of DNA (AGE)

6. Two-dimensional polyacrylamide gel electrophoresis

Media used for isolation and separation

Nowadays either an agarose gel or polyacrylamide gels are used.

Agarose gel

- Agarose- a linear polysaccharide (M.W. 12000 Da) made up of the basic repeat unit of agarobiose (which comprises alternating units of galactose and 3,6-anhydrogalactose.
- It is one of the components of agar, that is a mixture of polysaccharides from seaweeds.
- It is used at a concentration between 1% and 3%.

- Agarose gel is formed by suspending dry agarose in aqueous buffer and then boiling the mixture till it becomes clear solution, which is then poured and allowed to cool at room temperature to form rigid gel.
- The gelling properties is attributed to inter and intramolecular H-bonding within and between long agarose chains.
- The pore size of the gel is controlled by the initial concentration of agarose, large pore size corresponds to low concentration and vice versa.
- Although free from charges, substitution of the alternating sugar residues with carboxyl, methoxyl, pyruvate, and sulfate groups occur to varying degrees which can result in electroendosmosis during electrophoresis.
- Agarose is therefore sold in different purity grades, based on the sulfate concentration- the lower the sulfate concentration, the higher the purity.
- These gels are used for the electrophoresis of both proteins and nucleic acids.
- For proteins, the pore size of a 1% agarose gel is large relative to the sizes of proteins.
- Therefore, used in techniques such as immune-electrophoresis or flat-bed isoelectric focusing, where proteins are required to move unhindered in the gel matrix according to their native charge.
- Such large pure gels are also used to separate much larger molecules such as RNA and DNA, because the pore sizes are still large enough for RNA and DNA molecule to pass through the gel.
- An advantage of using agarose in the availability of low melting point agarose (62-65^{o}C).
- This gel can be reliquefied by heating to 65^{o}C and thus, for example DNA samples separated can be cut out of the gel, returned to solution and recovered.

Polyacrylamide gel

- Cross-linked polysaccharide gel is formed from the polymerization of acrylamide monomer in the presence of small amount of N,N'-methylene bis acrylamide (aka- bis-acrylamide).
- Bis-acryl amide is basically two acrylamide molecules linked by a methylene group, and is used as a cross-linking agent.
- Acrylamide monomers is polymerized in head to tail fashion into long chain, thus introducing a second site for chain extension.
- Proceeding in this way, a cross-linked matrix of fairly well-defined structure is formed.
- The polymerization of acrylamide is an example of free radical catalysis and is initiated by the addition of ammonium persulfate and the base N, N, N', N'- tetra-methylene diamine (TEMED).
- TEMED + Ammonium per sulphate(APS) for gel solidification

- Acrylamide gels are defined in terms of the total percentage of acrylamide present, and the pore size in the gel can be varied by changing the concentration of both acrylamide and bis-acrylamide.
- The acrylamide gel can be made with a content between 3% and 30% acrylamide.
- Thus, the low percentage gels (e.g., 4%) have large pore size and are used for electrophoresis of protein- example flatbed isoelectric focusing, or stacking gel system of an SDS-PAGE.
- Low percentage acrylamide gels are also used to separate DNA.
- Gels between 10% and 20% acrylamide are used in techniques such as SDS-gel electrophoresis, where smaller pore size now introduces a sieving effect that contributes to the separation of proteins according to their size.

Instrumentation of electrophoresis

1. Equipment required for electrophoresis basically consists of two items, a power pack and an electrophoresis unit.
2. Electrophoresis units are available for running either vertical or horizontal gel systems.
3. Vertical slab gel units are commercially available and routinely used to separate proteins in acrylamide gels.
4. The gel is formed between two glass plates, clamped together but held apart by plastic spacers.
5. Gel dimensions are typically 12cmX 14cm, with a thickness of 0.5 to 1mm.
6. A plastic comb is placed in the gel solution and is removed after polymerization to provide loading wells for samples.
7. When the apparatus is assembled, the lower electrophoresis, tank buffer surrounds the gel plates and affords some cooling of the gel plates.
8. The gel is cast on a glass or plastic plates and placed on a cooling plate (an insulated surface through which cooling water is passed to conduct away generated heat.)
9. Connection between the gel and electrode buffer is made using a thick wad of wetted filter paper, however the agarose gels for DNA electrophoresis are run submerged in the buffer.
10. The powerpack supplies a direct current between the electrodes in the electrophoresis unit.
11. All electrophoresis is carried out in an appropriate buffer, which is essential to maintain a constant state of ionization of the molecules being separated.
12. Any variation in pH would alter the overall charge and hence the mobilities (rate of migration in the applied field) of the molecules being separated.

Factor affecting electrophoresis:

- Nature of charge
- Voltage
- Frictional force
- Electrophoretic mobility

- Current
- Heat
- Elecrtroendosmosis

Applications of Electrophoresis

- Separation of biological molecules (DNA, RNA , protein)
- To visualize genomic DNA and Plasmid DNA
- To determine the digested size of proteins, genomic DNA , plasmid DNA and Plasmid forms
- To analyze the PCR products
- To analyze southern gel
- The ability of gene, protein transfer to gel to membrane is easier
- It used as preparative technique prior to use of other methods such as mass spectroscopy, cloning , DNA sequences, Southern Blotting for further characterization
- Separation of amino acid , lipoproteins and enzymes in blood.

ISOLATION OF GENOMIC DNA FROM BACTERIA

(Phenol/chloroform method)

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Aim

To isolate genomic DNA from bacterial cells and understand the general genomic pattern of a bacterium by phenol and chloroform method.

Background information

The genomic DNA isolation needs to separate total DNA from RNA, protein, lipid, etc. Initially the cell membranes must be disrupted in order to release the DNA in the extraction buffer. SDS (Sodium Dodecyl Sulphate) is used to disrupt the cell membrane. Once cell is disrupted, the endogenous nucleases tend to cause extensive hydrolysis. Nucleases apparently present on human fingertips are notorious for causing spurious degradation of nucleic acids during purification.

DNA can be protected from endogenous nucleases by chelating Mg^{2++} ions using EDTA. Mg^{2++} ion is considered as a necessary cofactor for action of most of the nucleases. Nucleoprotein interactions are disrupted with SDS, phenol or proteinase K. Proteinase enzyme is used to degrade the proteins in the disrupted cell soup.

Phenol and chloroform are used to denature and separate proteins from DNA. Chloroform is also a protein denaturant, which stabilizes the rather unstable boundary between an aqueous phase and pure phenol layer.

The denatured proteins form a layer at the interface between the aqueous and the organic phases which are removed by centrifugation. DNA released from disrupted cells is precipitated by cold absolute ethanol or isopropanol.

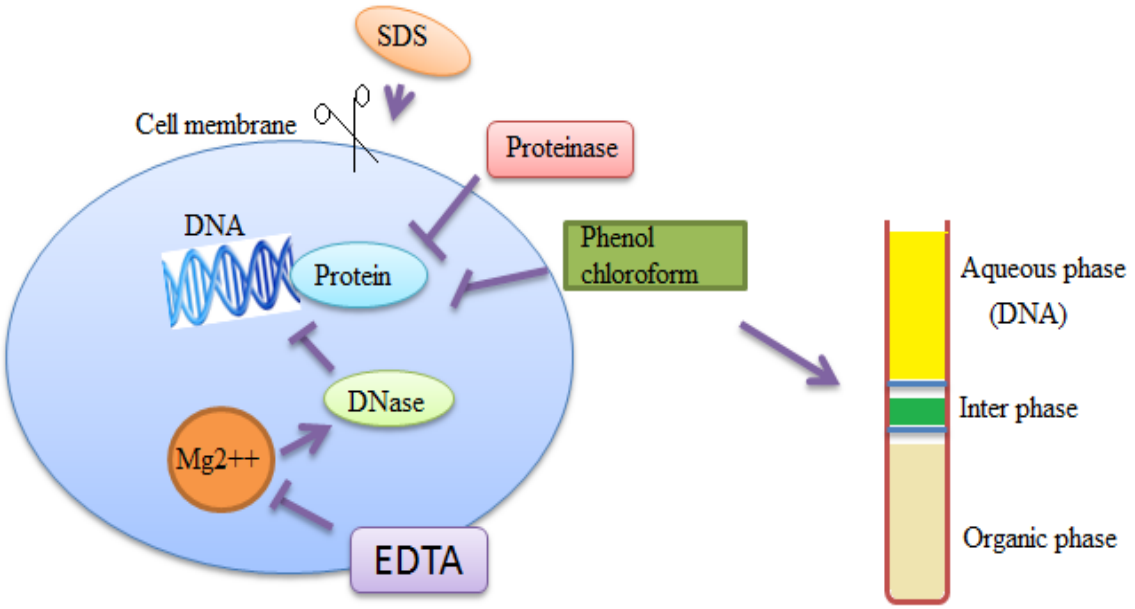

Figure 1: Schematic diagram showing the principle of isolation of genomic DNA from cell

Materials and Reagents Required

1. Tris base
2. Proteinase K
3. Phenol\chloroform (1: 1)
4. 200 proof ethanol
5. RNAase
6. Ethanol
7. SDS
8. EDTA
9. Tryptone
10. Yeast extract
11. NaCl
12. LB medium - 1% tryptone, 0.5% yeast extract, 200 mM NaCl
13. TE buffer - 10 mM Tris-Cl (pH 8.0), 1 mM EDTA (pH 8.0)
14. Lysis buffer - (10 ml) 9.34 ml TE buffer, 600 ul of 10% SDS, 60 µl of proteinase K (20 mg ml^{-1})

Equipment Required

1. Tabletop centrifuge (Eppendorf)
2. 1.5 ml Eppendorf tube
3. Incubator
4. Gloves

Procedure

1. Transfer 1.5 ml of the overnight *E. coli* culture (grown in LB medium) to a 1.5 ml Eppendorf tube and centrifuge at max speed for 1min to pellet the cells.
2. Discard the supernatant. *Note: Remove as much of the supernatant as you can without disturbing the cell pellet.*
3. Resuspend the cell pellet in 600 µl lysis buffer and vortex to completely resuspend cell pellet.
4. Incubate 1 h at 37 °C.
5. Add an equal volume of phenol/chloroform and mix well by inverting the tube until the phases are completely mixed. *Note: Do not vertex the tube—it can shear the DNA.*
   CAUTION: Phenol is a very strong acid that causes severe burns. Chloroform is a carcinogen. Wear gloves, goggles and lab coat, and keep tubes capped tightly. To be safe, work in the hood if possible.
6. Spin at max speed for 5 min at RT (all spins are performed at RT, unless indicated otherwise). There is a white layer (protein layer) in the aqueous: phenol/chloroform interface.
7. Carefully transfer the upper aqueous phase to a new tube by using 1 ml pipetman (to avoid sucking the interface, use 1 ml tip with wider mouth-cut 1 ml tip-mouth about ~2 mm shorter).
8. Steps 4-6 can be repeated until the white protein layer disappears.
9. To remove phenol, add an equal volume of chloroform to the aqueous layer. Again, mix well by inverting the tube.
10. Spin at max speed for 5 min.
11. Remove aqueous layer to new tube.
12. To precipitate the DNA, add 2.5 or 3 volume of cold 200 proof ethanol (store ethanol at -20 °C freezer) and mix gently (DNA precipitation can be visible). *Note: DNA precipitation may simply diffuse, which is normal. Keep the tube at -20 degree for at least 30 min (the longer the better) and then spin it down (see Steps 15-16). You should see DNA pellet. It looks transparency when it is wet and turns to white when it becomes dry.*
13. Incubate the tube at -20 °C for 30 min or more.
14. Spin at max speed for 15 min at 4 °C.
15. Discard the supernatant and rinse the DNA pellet with 1 ml 70% ethanol (stored at RT).
16. Spin at max speed for 2 min. carefully discard the supernatant and air-dry the DNA pellet (tilt the tube a little bit on paper towel). To be faster, dry the tube at 37 °C incubator.

17. Resuspend DNA in TE buffer. *Note: Large amounts of RNA will be present in the DNA sample. So, for subsequent reactions, for example, to digest plasmid DNA, add 1-5 µl (1 mg ml^{-1}) RNAase to the digestion solution to completely remove RNA. Or, add RNAase directly to lysis buffer with a final concentration of 1 mg ml^{-1}.*

18. Check isolated Gemonic DNA on an agarose gel. *Note: we expect to see bands with smear patterns from high to low MW range, although most of DNA fragments are accumulated at high MW on the gel. So, if you see most of DNA fragments are small, very likely your DNA got degraded.*

SEPARATION OF BACTERIAL GENOMIC DNA USING AGAROSE GEL ELECTROPHORESIS

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Aim

To separate and visualize different size of DNA fragments through agarose gel matrix and an electrical current.

Background information

Fragments of DNA can be separated by electrophoresis through agarose gel. Agarose is the derivative of the agar, a polysaccharide isolated from seaweed. When a hot solution of agarose cools, the polysaccharide strands fold and twist around each other forming mesh on gel. When a current is applied the negative charged DNA molecule move to the gel towards the positive cathode. Small sized fragment of DNA move through the gel, move quickly than the larger size fragment. The result is that DNA fragment separate on the basis of the size.

One of the important advantages of agarose is that the gel has very large pore size and relatively low level of fixed electrical charge and electrode osmosis. (the flow of liquid towards the cathode created by the protein of fixed negative charges in the supporting gel). One common way to visualized DNA in agarose gel is by staining with EtBr. The dye was fluoresces orange when expressed to UV light. EtBr is a flat, planner molecules that intercalated and slight between the stalked between base pairs of DNA. Since the flurescent emitted by EtBr bound to DNA is than that of unbound dye small amount of DNA can be detected by presence of EtBr. If DNA molecular weight known strands which are linear fragment of DNA of known size are run on the same gel, then the sizes of the other fragment of DNA can be determined.

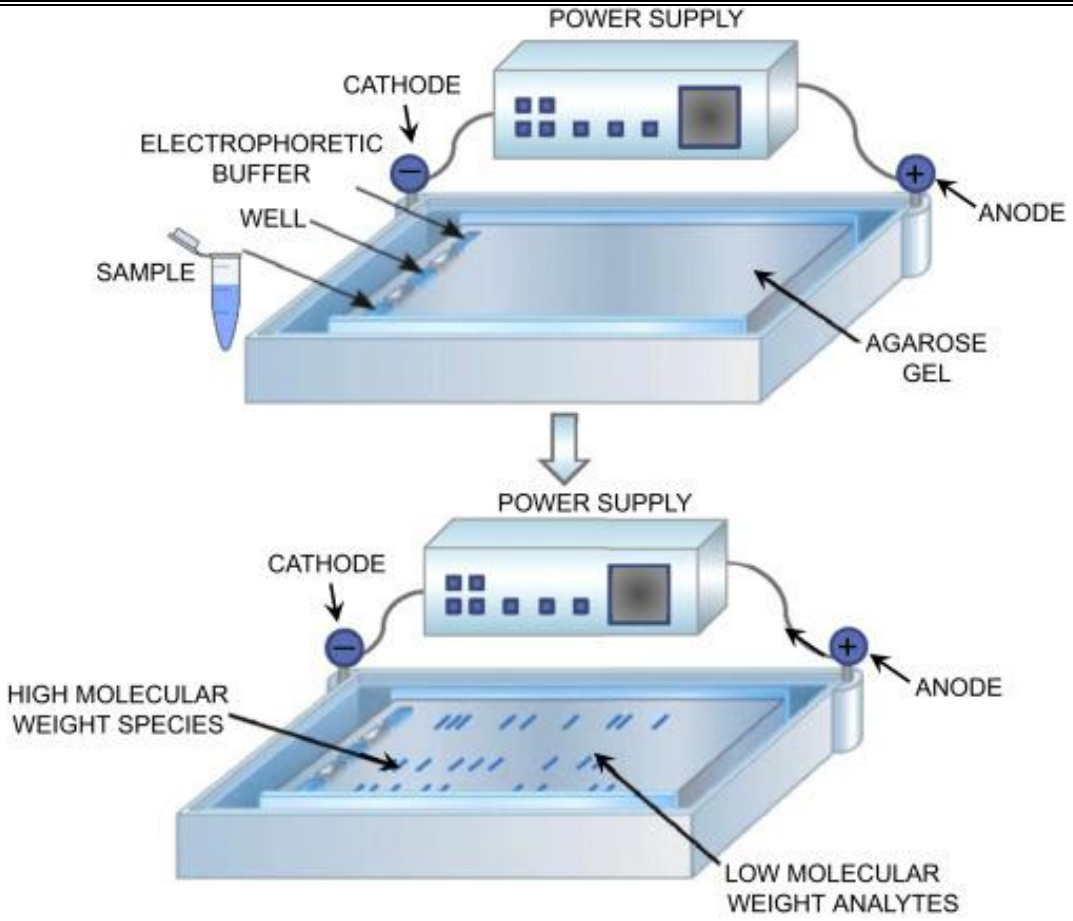

Figure 2: Agarose electrophoresis system

Materials Required

- 1% Agarose
- Sample Buffer
- Glycerol
- TAE buffer
- 0.5M EDTA
- Bromophenol blue
- EtBr
- 5× TAE
- Tris Base
- Boric Acid
- Dipottasium EDTA

Procedure

- The gel tray was cleaned with distilled water and wiped with ethanol; the gel tray was sealed with cellophane tape.

- Sufficient amount of agarose was melted in 1× TAE buffer, the solution was allowed to cooled down to bearable temperature and approximately EtBr was added and mixed thoroughly.
- The comb was carefully removed from the gel and the gel was immersed in electrophoresis tank containing the buffer.
- 18µl of sample was mixed with 2 µl of loading dye and load into the well using micropipette.
- The electrodes were connected and gel was electrophorsed at 60-80V. The run was continued for 1 hour.
- After running the gel was viewed in UV transilluminator.
- The gel photograph was captured with gel documentation.

Result

After agarose gel electrophoresis the Nucleic acid bands were characterized using molecular weight marker.

Discussion

The isolated genomic and plasmid DNA were confirmed by using Agarose Gel Electrophoresis. In agarose gel, the DNA was observed as an orange colour band under UV-transilluminator due to the addition of ethidium bromide in gel an intercalating agent which intercalates with DNA bases.

BASICS ON *INSILICO* TOOLS

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1. Introduction

The term ‘in-silico' is derived from the Latin word “Insilicon”. It refers that “the experimentation performed by computer and is related to the more established terms of in-vivo and in-vitro”. It covers the development of techniques for using software to capture, analyse and integrate biological and medical data from many diverse sources. More specifically, it defines the use of biological data this information in the creation of computational models or simulations that can be used to make predictions, suggest hypotheses, and ultimately provide discoveries or advances in biological sciences. medicine and therapeutics.

In 1989, Pedro Miramontes, in his presentation, DNA and RNA Physicochemical Constraints, Cellular Automata and Molecular Evolution at the workshop on Cellular Automata: Theory and Applications, used the term in-silico to represent biological experiments carried out in computers.

In silico studies finds its wide applications initially in the field of understanding the interaction of drugs with body and pathogens. Kinnings*et al.* (2009) predicted the use of commercial drugs against MDR and drug resistant strains of TB using software emulations.

In-silico methods are include databases, quantitative structure-activity relationships, similarity searching pharmacophores, homology models and other molecular modeling, machine learning, data mining, network analysis tool and data analysis tools that use a computer, but some important techniques are discussed below:

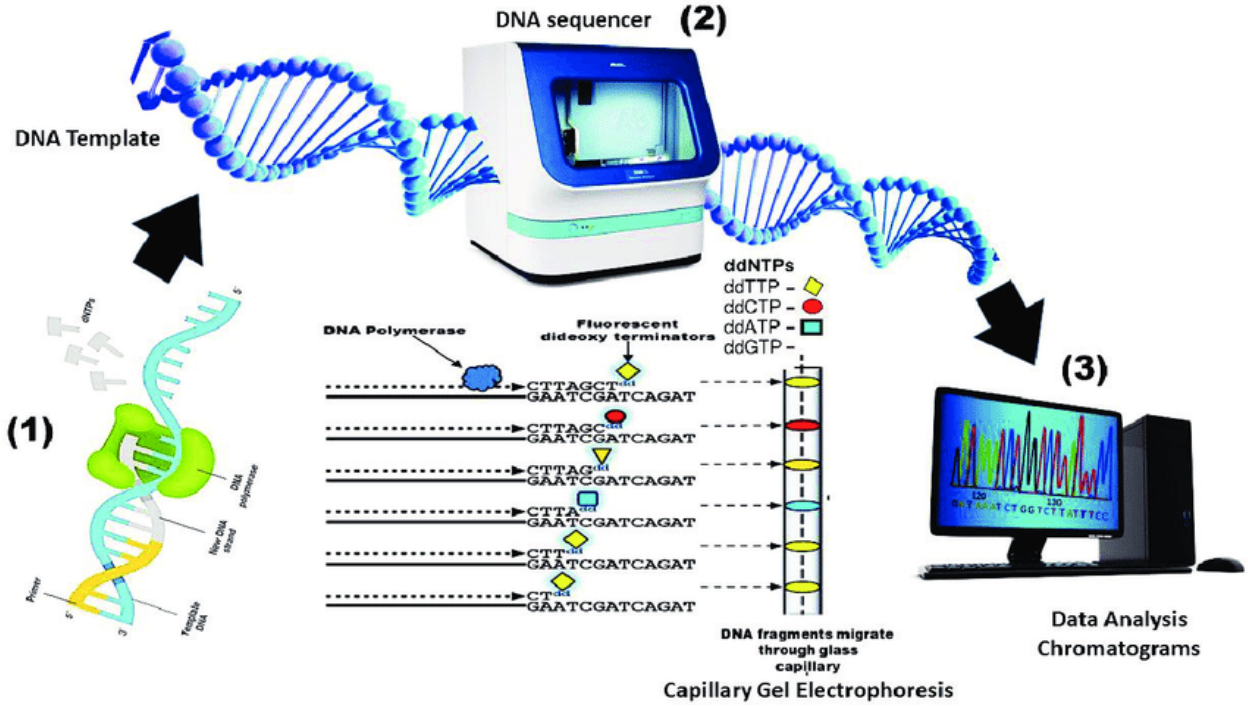
2. Basics of microbial DNA sequencing and submission it to repository

2a. DNA sequencing method

As an alternative to in vitro methods for identifying bacteria, various in-silico methods which sequence bacterial DNA and RNA have been developed. The most commonly technique is polymerase chain reaction (PCR). PCR takes a single or few copies of a piece of DNA and amplifies increases it across several orders of magnitude, generating millions or more copies of a particular DNA sequence.

The term DNA sequencing refers to sequencing methods for determining the order of the nucleotide bases- adenine, guanine, cytosine and thymine in a molecule of DNA. It enables us to perform a thorough analysis of DNA as it provides us with the most basic information of the nucleotides. Scientists recognized that this could potentially be a very powerful tool. Than in 1974, two techniques for sequencing DNAwere independently developed by an American team and English team to do exactly this. The Americans, lead by Maxam and Gilbert, adoptingused a chemical cleavage protocol and while the English, lead by Sanger, designed a with a protocol similar to the natural process of DNA replication. Eventhough both team of Scientists shared the 1980 Nobel Prize, Sanger's method became the standard because of its practicality.

Sanger method is based on utilization of DNA polymerase, di-deoxynucleotides, and different dNTPs. Here the di-deoxynucleotide terminates the chain reaction of DNA synthesis which produces the different length of chains. This method involves the following steps: a) Four reaction tubes are set up each containing single stranded DNA sample to be sequenced, all four dNTPs (radioactively labelled) and an enzyme for DNA synthesis (DNA polymerase I); b) Each tube also contains a aliquot small amount of (much smaller amount relative to four dNTPs) one of the ddNTP, so that four tubes have each different ddNTP, in such a way that each tube containing different ddNTP bringing about termination at a specific base -Adenine (A), Cytosine (C), Thymine (T), and Guanine (G); c) The fragments generated by random incorporation of ddNTP leads to termination of reaction and so the different fragments are produced which can be separated by high resolution polyacrylamide gel, four adjoining lanes are loaded by four different samples; d) The gel is then autoradiographed, the position of different bands in each lane can be visualized, and based on the position of the bands, the DNA sequence can be read out very easily. This method, as usual, the sequencing primer is labelled with ^{32}P and the mixtures with amplified DNA, Taq polymerase and appropriate buffer are incubated at 70°C for 5 min. The reaction is stopped by addition of formamide and mixtures are allowed to run polyacrylamide sequencing gel, which can be read by computer or manually.



Source:Ahmed Shuikan et al. 2019

2b. How to submission of DNA sequence to repository

After sequencing the DNA sequence, it has to be the same may be submitted to a public sequence repository (DDBJ/ENA/Genbank - INSDC) as part of the publication process. Data exchange between DDBJ, ENA and GenBank occurs daily so it is enough only necessary to submit the sequence to anyone of the databases, whichever one is most convenient, without regard for where the sequence may be published. GenBank will provide accession numbers for submitted sequences. This accession number serves as an identifier for your submissions your data, and allows the community to retrieve the sequence upon reading the journal article. The accession number will be useful for publication of article.

3. Sequence analysis – Database similarity searches

3a. Sequence analysis

The sequence analysis refers to the process of subjecting a DNA, RNA or peptide sequence to any of a wide range of analytical methods to understand its features, function, structure, or evolution.

Methodologies used include sequence alignment, searches against biological databases, and others. Since the development of methods of high-throughput production of gene and protein sequences, the rate of addition of new sequences to the databases increased exponentially. It can be used to assign function to genes and proteins by the study of the similarities between the compared sequences.

Database searches usually provide the first clues of whether the sequence is similar to the sequence already studied and well known protein family. If there is similarity to a sequence that is from another species, then they may be homologous. (i.e., Sequences that descended from a common ancestral sequence). Knowing the function of a similar / homologous sequence will often give a good indication of the identity of the unknown sequence.

3b. NCBI

National Center for Biotechnology Information (NCBI) is one of the leading online resources known for providing Biological sequence information. NCBI is maintained by two organizations in US, National Library of Medicine (NLM) and National Institute of science (NIH). As a national resource for molecular biology information, NCBI's mission is to develop new information technologies to aid in the understanding of fundamental molecular and genetic processes that control health and disease.

Biological databases are an important tool in assisting scientists to understand and explain a host of biological phenomena from the structure of biomolecules and their interaction, to the whole metabolism of organisms and to understanding the evolution of species. This knowledge helps facilitate tothe fight against diseases, assists in the development of medications and in discovering basic relationships amongst species in the history of life.

Biological knowledge is distributed amongst many different general and specialized databases. This sometimes makes it difficult to ensure the consistency of information. Biological databases cross-reference other databases with accession numbers as one way of linking their related knowledge together.

Biological database has been classified into Sequence and Structural databases. A sequence database contains nucleotide and protein sequence information. There are currently three main public Nucleotide databases:

EMBL: The European Molecular Biology Laboratory, Cambridge, UK.

GenBank: NCBI, a division of NLM at the NIH campus, USA.

DDBJ: The DNA databank of Japan.

The protein databases are:

SWISSPROT & UNIPROT: Protein sequence databases

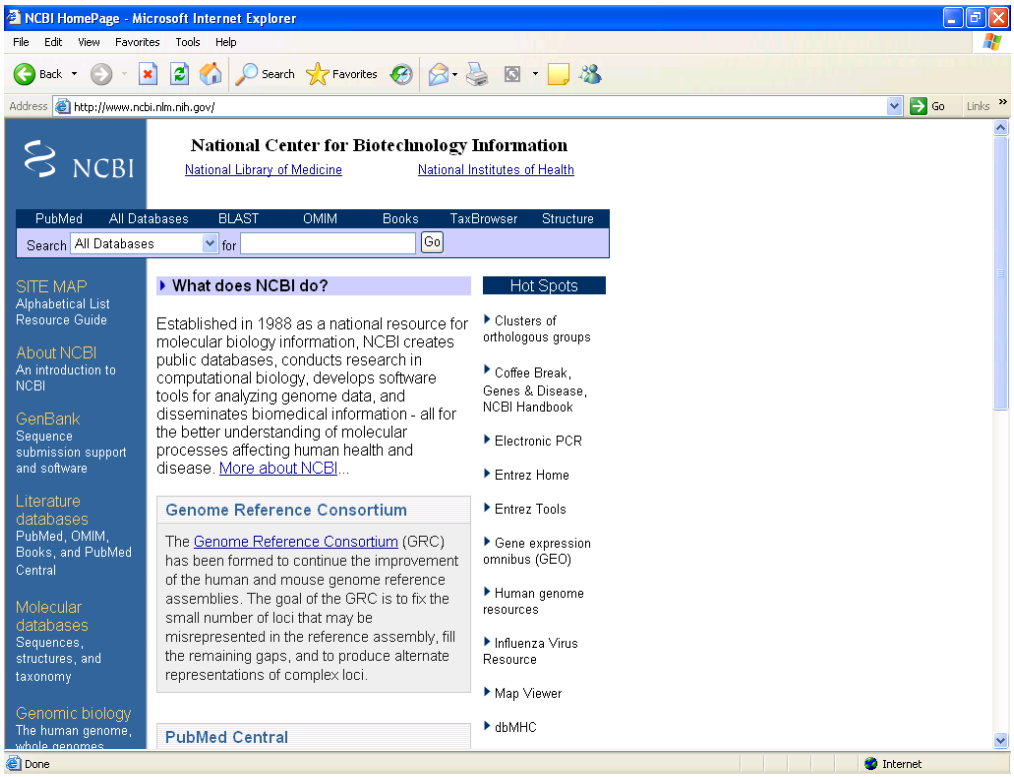
PIR: Protein Information Resources

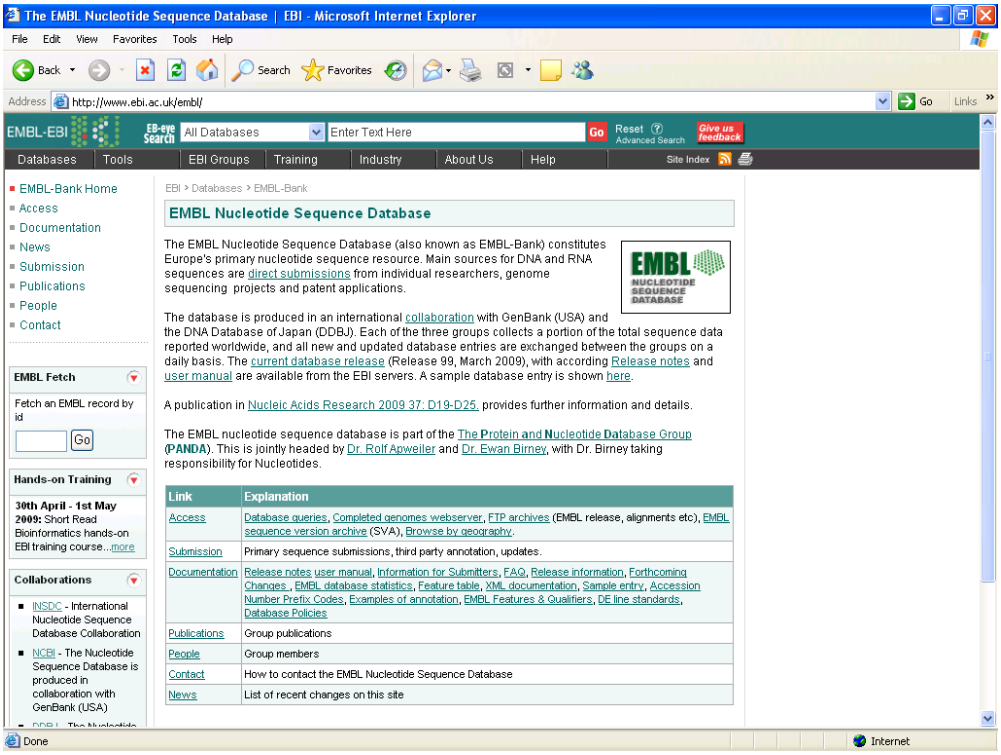
PDB: Protein Databank

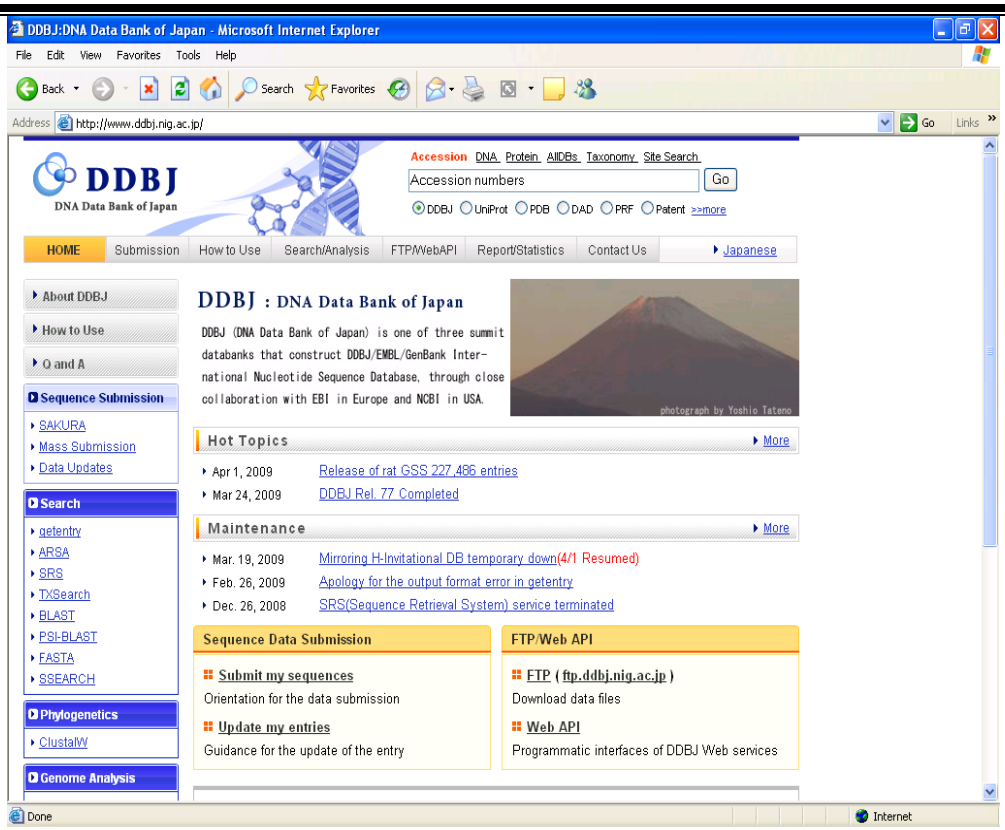
As these databases contain hundreds of thousands of sequences, searching through them requires the processing power of a computer search engine. The Sequence Retrieval System (SRS) has been designed to do the search just that. SRS is available at many sites acrossover the world. However, every site allows access to a different set of databases and sometimes, search and analysis tools. If you are doing more work with proteins, you might want to investigate the proteomics server namely, Expert Protein Analysis System (ExPASy) located held at the Swiss Institute of Bioinformatics which is proteomics server.

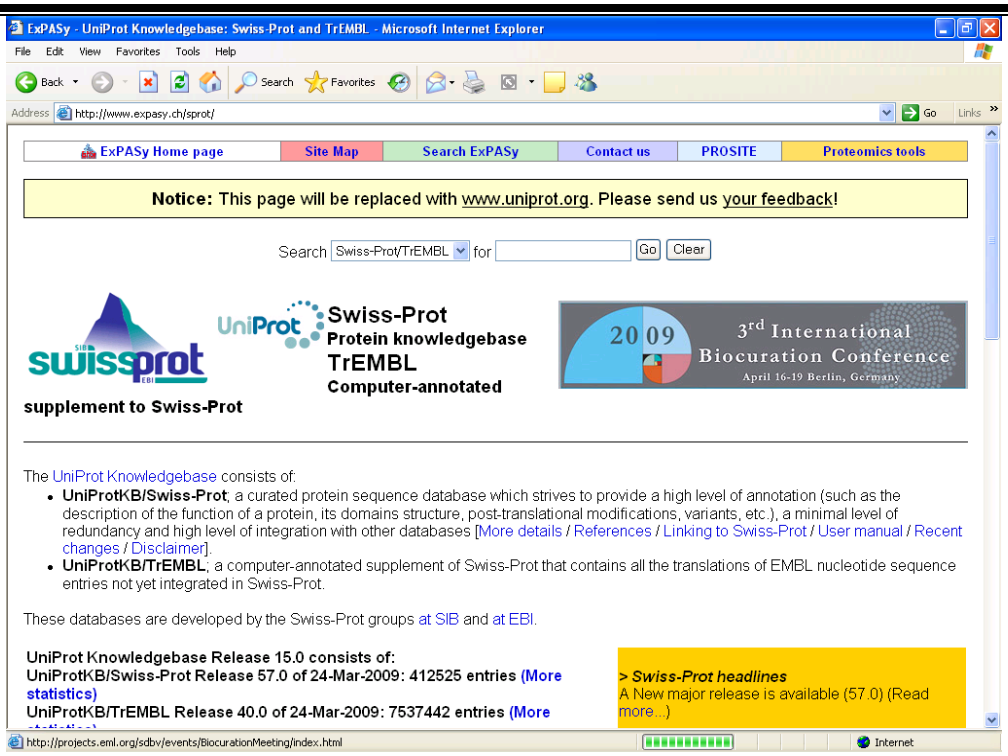
ProtParam is a tool which allows the computation of various physical and chemical parameters for a user entered protein sequence. No additional information is required about the protein under consideration. The protein can either be specified as a Swiss – Prot / TrEMBL accession number or ID, or in form of a raw sequence. White space and numbers are ignored. If you provide the accession number of a Swiss – Prot / TrEMBL entry, you will be prompted with an intermediary page that allows you to select the portion of the sequence on which you would like to

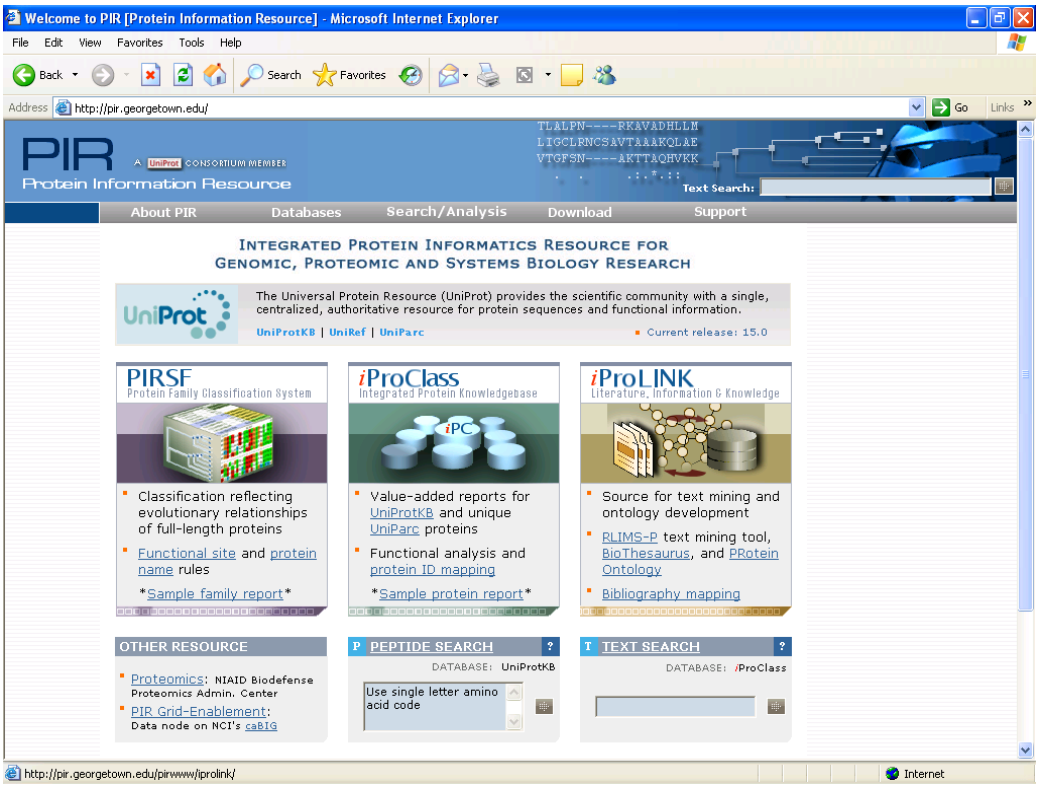
perform the analysis. The choice includes a selection of mature chains or peptides and domains form the Swiss – Prot feature table (which can be chosen by clicking on the positions), as well as the possibility to enter start and end position in two boxes. By default (ie. If you leave the two boxes empty) the complete sequence will be analysed. The computed parameters include the molecular weight, theoretical pH, aminoacid composition, atomic composition, extinction coefficient, estimated half –life, instability index, aliphatic index and grand average of hydropathicity.

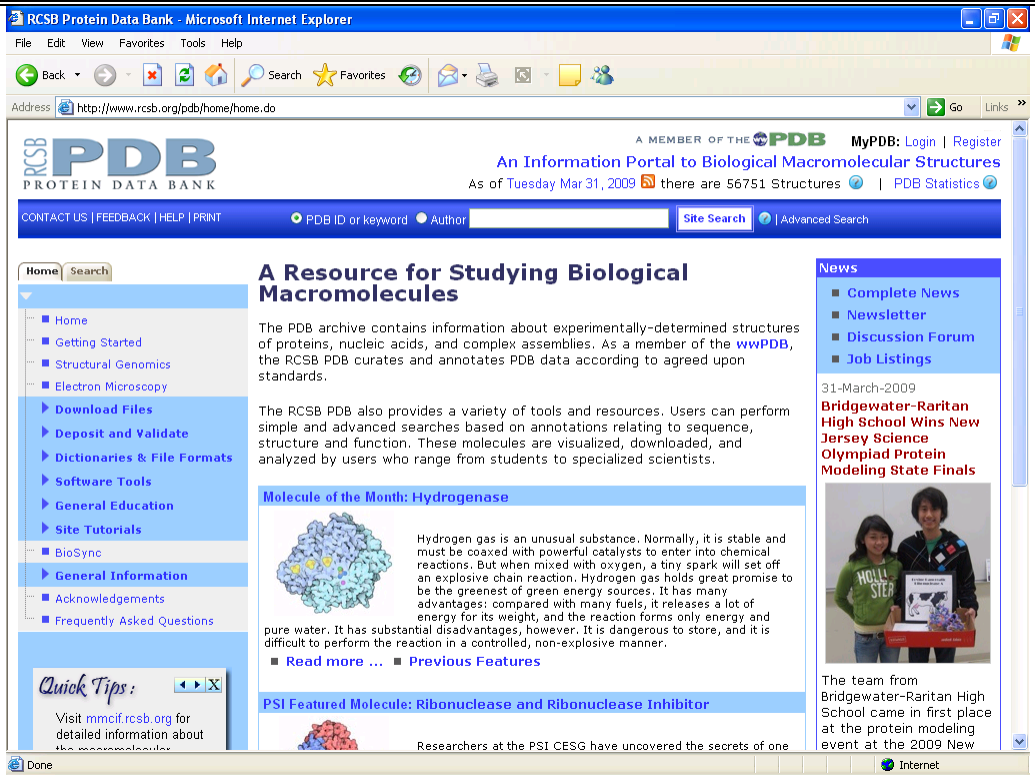





DDBJ:DNA Data Bank of Japan - Microsoft Internet Explorer
File Edit View Favorites Tools Help
Back Search Favorites
Address http://www.ddbj.nig.ac.jp/
Go Links
DDBJ
DNA Data Bank of Japan
Accession DNA Protein AllDBs Taxonomy Site Search
Accession numbers
Go
DDBJ UniProt PDB DAD PRF Patent >>more
HOME Submission How to Use Search/Analysis FTP/WebAPI Report/Statistics Contact Us Japanese
About DDBJ
How to Use
Q and A
Sequence Submission
SAKURA
Mass Submission
Data Updates
Search
getentry
ARSA
SRS
TXSearch
BLAST
PSI-BLAST
FASTA
SSEARCH
Phylogenetics
ClustalW
Genome Analysis
DDBJ : DNA Data Bank of Japan
DDBJ (DNA Data Bank of Japan) is one of three summit databanks that construct DDBJ/EMBL/GenBank International Nucleotide Sequence Database, through close collaboration with EBI in Europe and NCBI in USA.
photograph by Yoshio Tateno
Hot Topics More
Apr 1, 2009 Release of rat GSS 227,486 entries
Mar 24, 2009 DDBJ Rel. 77 Completed
Maintenance More
Mar. 19, 2009 Mirroring H-Invitational DB temporary down(4/1 Resumed)
Feb. 26, 2009 Apology for the output format error in getentry
Dec. 26, 2008 SRS(Sequence Retrieval System) service terminated
Sequence Data Submission
Submit my sequences
Orientation for the data submission
Update my entries
Guidance for the update of the entry
FTP/Web API
FTP (ftp.ddbj.nig.ac.jp)
Download data files
Web API
Programmatic interfaces of DDBJ Web services
Done
Internet


ExPASy - UniProt Knowledgebase: Swiss-Prot and TrEMBL - Microsoft Internet Explorer
http://www.expasy.ch/sprot/
ExPASy Home page
Site Map
Search ExPASy
Contact us
PROSITE
Proteomics tools
Notice: This page will be replaced with www.uniprot.org. Please send us your feedback!
Search Swiss-Prot/TrEMBL for Go Clear
Swiss-Prot
Protein knowledgebase
TrEMBL
Computer-annotated
supplement to Swiss-Prot
2009 3rd International Biocuration Conference
April 16-19 Berlin, Germany
The UniProt Knowledgebase consists of:
UniProtKB/Swiss-Prot; a curated protein sequence database which strives to provide a high level of annotation (such as the description of the function of a protein, its domains structure, post-translational modifications, variants, etc.), a minimal level of redundancy and high level of integration with other databases [More details / References / Linking to Swiss-Prot / User manual / Recent changes / Disclaimer].
UniProtKB/TrEMBL; a computer-annotated supplement of Swiss-Prot that contains all the translations of EMBL nucleotide sequence entries not yet integrated in Swiss-Prot.
These databases are developed by the Swiss-Prot groups at SIB and at EBI.
UniProt Knowledgebase Release 15.0 consists of:
UniProtKB/Swiss-Prot Release 57.0 of 24-Mar-2009: 412525 entries (More statistics)
UniProtKB/TrEMBL Release 40.0 of 24-Mar-2009: 7537442 entries (More
> Swiss-Prot headlines
A New major release is available (57.0) (Read more...)
http://projects.eml.org/sdbv/events/BiocurationMeeting/index.html
Internet


Welcome to PIR [Protein Information Resource] - Microsoft Internet Explorer
http://pir.georgetown.edu/
PIR
Protein Information Resource
A UniProt CONSORTIUM MEMBER
Text Search:
About PIR
Databases
Search/Analysis
Download
Support
INTEGRATED PROTEIN INFORMATICS RESOURCE FOR GENOMIC, PROTEOMIC AND SYSTEMS BIOLOGY RESEARCH
UniProt
The Universal Protein Resource (UniProt) provides the scientific community with a single, centralized, authoritative resource for protein sequences and functional information.
UniProtKB | UniRef | UniParc
Current release: 15.0
PIRSF
Protein Family Classification System
Classification reflecting evolutionary relationships of full-length proteins
Functional site and protein name rules
Sample family report
iProClass
Integrated Protein Knowledgebase
Value-added reports for UniProtKB and unique UniParc proteins
Functional analysis and protein ID mapping
Sample protein report
iProLINK
Literature, Information & Knowledge
Source for text mining and ontology development
RLIMS-P text mining tool, BioThesaurus, and PRotein Ontology
Bibliography mapping
OTHER RESOURCE
Proteomics: NIAID Biodefense Proteomics Admin. Center
PIR Grid-Enablement: Data node on NCI's caBIG
PEPTIDE SEARCH
DATABASE: UniProtKB
Use single letter amino acid code
TEXT SEARCH
DATABASE: iProClass
http://pir.georgetown.edu/pirwww/iprolink/
Internet



3c. Retrieval of the nucleotide sequence of microbial gene from GenBank.

The GenBank sequence database is an open access, annotated collection of all publicly available nucleotide sequences and their protein translations.

STEPS

1. Open the NCBI webpage http://www.ncbi.nih.nlm.gov/ from google search page.
2. Searches related to NCBI will be displayed.
3. Select the nucleotide search tool from all database option in genbank.
4. Type the specific nucleotide (eg. Microbial gene or enzyme) name into the search box and click the go button to perform search.
5. The tool displayed few list of species containing the nucleotide sequence.
6. Select a specific organism from that set to retrieve the nucleotide sequence in FASTA format and GenBank format

Flat file format

The flat file contains 3 divisions: Header, Feature table and Nucleotide sequence.

1. The Header

*LOCUS:*Introduction of a short label for the entry line summarizes other relevant facts including the number of bases, source of sequence date, section of database and date of submission

DEFINITION: This line contains a concise description of sequence.

ACCESSION NUMBER: This line gives the accession number, a unique constant code assigned to each entry.

VERSION: This line indicates the version of any sequence revisions, also include a nucleotide identifier.

KEYWORD: This line introduces a list of short phrases assigned by the author, describing, gene product and other relevant information about entry.

SOURCE: Record provides information on the source from which the data have been divided.

ORGANISM: Illustrates the biological classification of the source organisms.

REFERENCE: Records indicate the protein of sequence data to which the literature refers.

AUTHOR, TITLE, JOURNAL: Provides a structure for the citation.

MEDLINE: It is a point to a online medical literature information resource, which allows the abstract of a given article to be viewed

COMMENT: It is the free text section that allows any additional annotation to be added to the entry.

2. The Feature Table

FEATURE: It marks the feature table, whose purpose is to describe the properties of the sequence in detail, such as the gene name, coordinates of its coding sequences.

CODINGSEQUENCE: Contains instruction to the reader on how to join two sequences together or how to make an amino acid sequence from the indicated coordination and the intend genetic code.

BASECOUNT: Gives the details of the frequency of the different base types in the sequences.

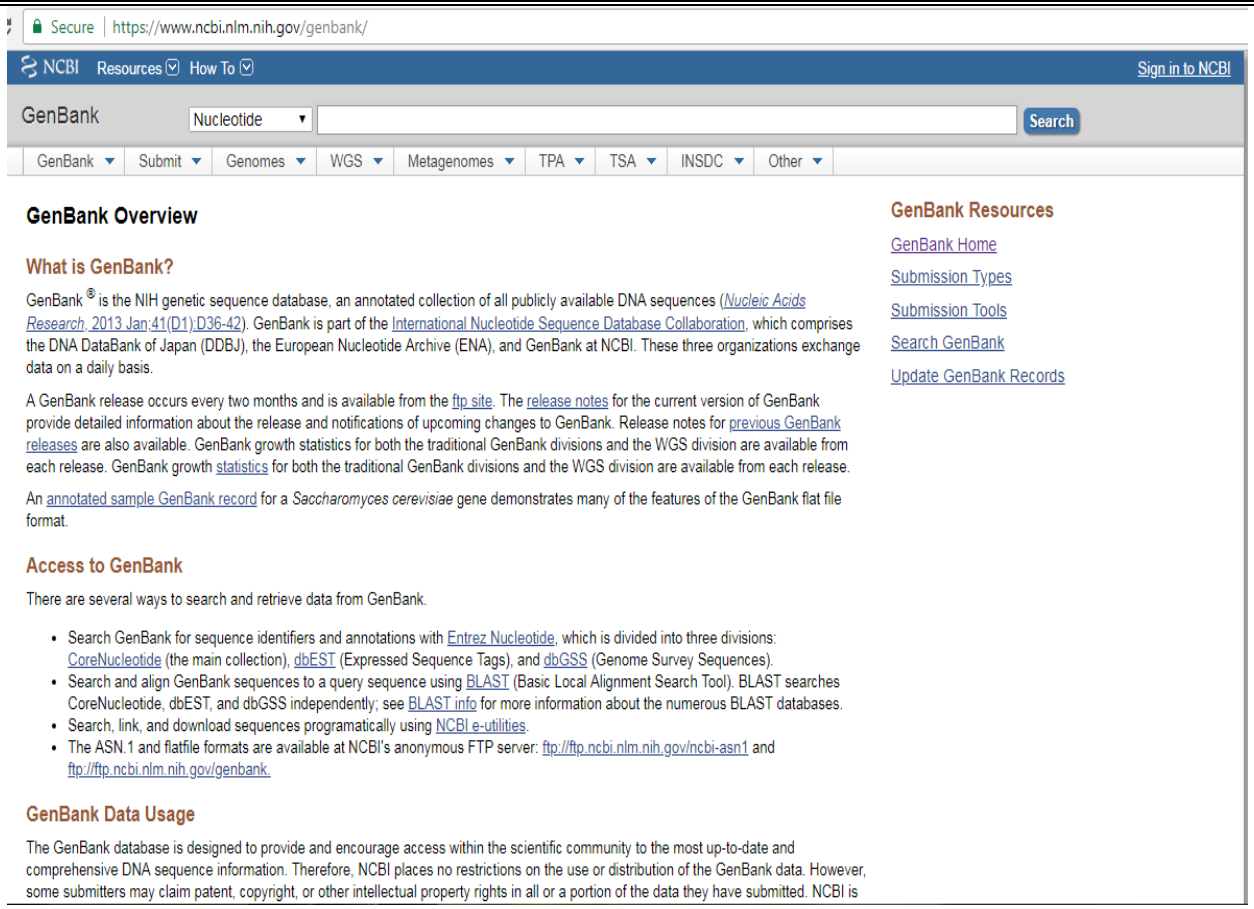
3.Nucleotide Sequences

ORIGIN: This line notes, where possible the location of the first base of the sequence in the genome. The nucleotide sequence itself follows.

// flat file format always end with double forward slash.

Result

Home page of NCBI



Flat File Format of NCBI

ORIGIN

1 ggaggctcctccctgggctacgggggcctgtatggctatggaggctcctccctgggctac

61 gggggcctgtatggctatggaggctcctccctgggctacgggggcctgtatggctatggt

121 agatcctatggttccggctactgcagcccttactcctaccggtacaacaggtaccgccgt

181 ggcagctgcgggccctgctaaattaaaagtttattgcatc//

4. Pair wise sequence alignment by using BLAST (Basic Local Alignment Search Tool)

BLAST is the main NCBI tool for comparing a protein or DNA sequence to other sequences, in various databases. BLAST searching is one of the fundamental ways of learning of interested or target protein or gene. The search reveals what related sequences are present in the same algorithm and other organism.

BLAST searching allows the user, to select one sequence (termed the query) and perform pairwise sequence alignment between the query and an entire database (termed the target). The Needleman – Wunsch (1970) and Smith Watermann (1980) are referred as local and global alignment algorithms respectively.

BLAST (Basic Local Alignment Search Tool), uses the method of Altschel*et al.* to search for similarities between a query sequence and all the sequences in a database. We can specify any number of query sequences to BLAST, and they may be in any combination of protein or nucleotide

sequences. BLAST searches one or more nucleic acid or protein databases for sequences similar to one or more query sequences of any type. BLAST can produce alignments for the matches it finds.

BLAST is a family of programmes that allows, use to input a query sequence and compare it to DNA or protein sequences in a database. The programs produce high scoring segment pairs that represent local alignments between your sequence and database sequences.

Five different programs in the BLAST family:

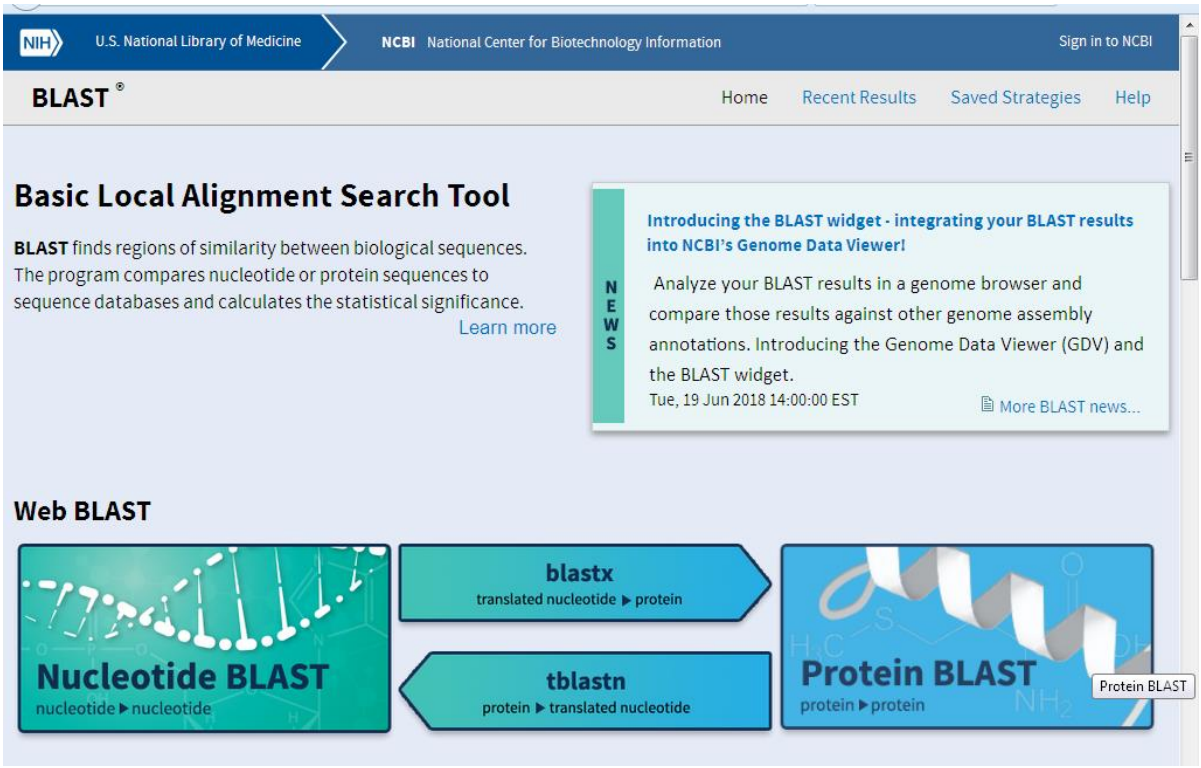
- **BLASTN** (Nucleotide query searching a Nucleotide Database)
  Each nucleotide database sequence is compared to a query in a separate nucleotide – nucleotide pairwise comparisons.
- **BLASTP** (Protein Query searching a Protein Database)
  Each database sequence is compared to each query in a separate protein – protein pairwise comparison.
- **BLASTX** (Nucleotide Query Searching a Protein Database)
  Each query is translated, and each of the six products is compared to each database sequence in separate protein – protein pairwise comparison.
- **TBLASTN** (Protein query searching a Nucleotide Database)
  Each Nucleotide database sequence is translated, and each of the six products is compared to the queries in a separate protein – protein pairwise comparison.
- **TBLASTX**(Nucleotide query searching a Nucleotide Database)
  The query and database sequences are translated in six frames, and each of the 12 products (for each query sequence) is compared in 36 different pairwise comparisons. Because this program involves more computation than the others, gapped alignments are not available when using TBLASTX.

Steps of Nucleotide sequence alignments as are follows:

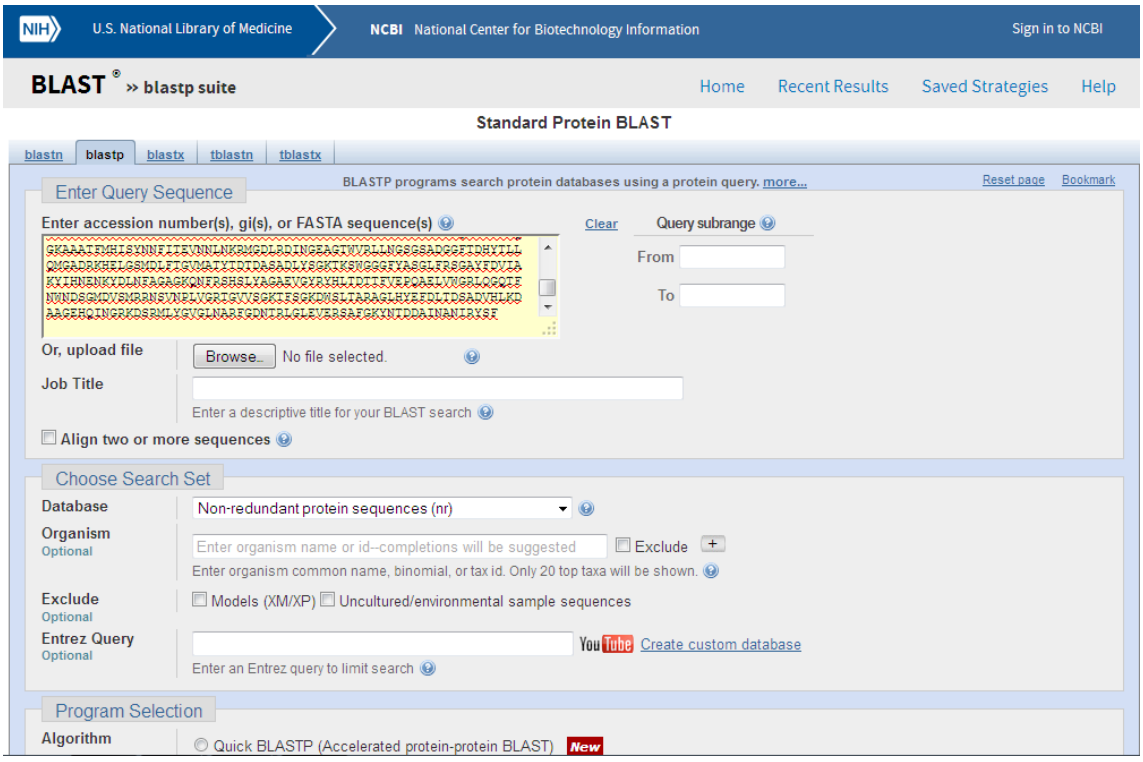
1. Open the BlastN program page from NCBI (www.ncbi.nlm.nih.gov)
2. Retrieve the nucleotide sequence from the NCBI.
3. Paste the retrieved nucleotide sequence in FASTA format in the field titled "Enter Query Sequence" or Click on "Browse" and select the sequence.
4. Enter a Job Title, if necessary.
5. In the "Choose Search Set" section, change the database to "Reference mRNA
6. sequences (refseq_rna)".
7. Under "Program Selection", select "Somewhat similar sequences (blastn)"
8. Check the box "Show results in a new window" next to the "BLAST" button and click "BLAST".
9. BLAST will now open a new window and shows it is working on your search.
10. Once the results are computed they will be presented in the window.

Results:

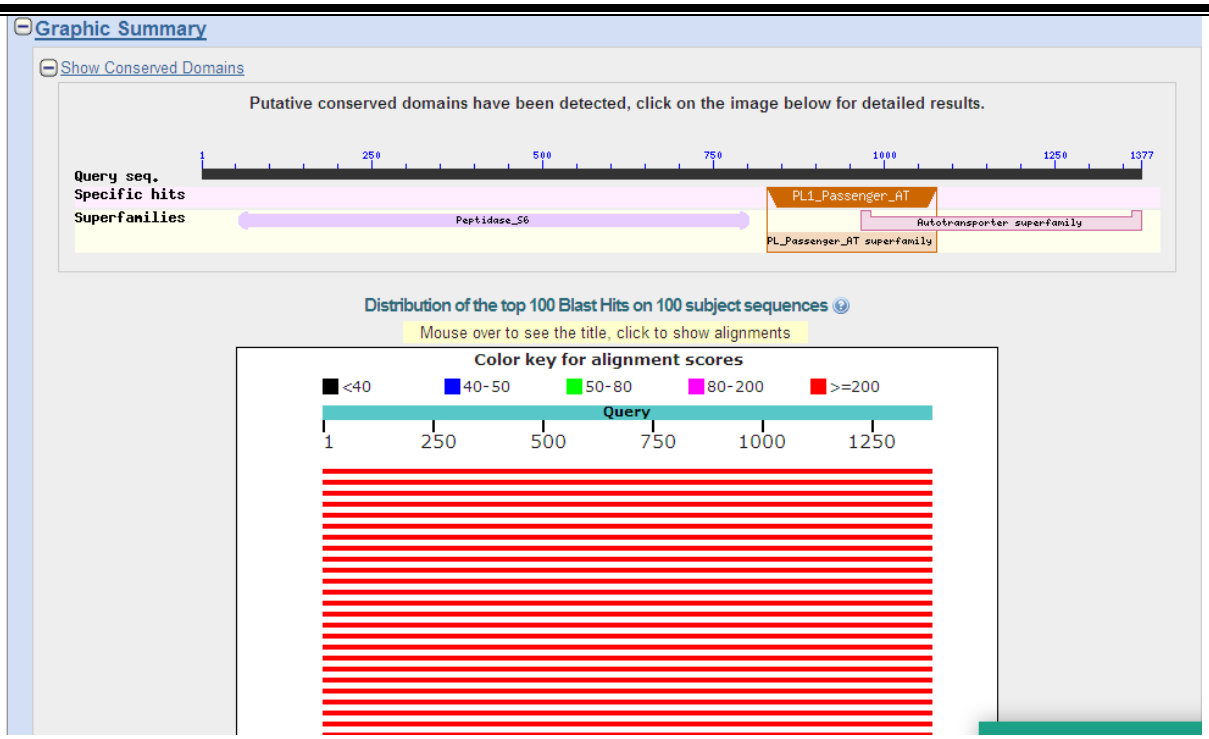
Home page of BLAST

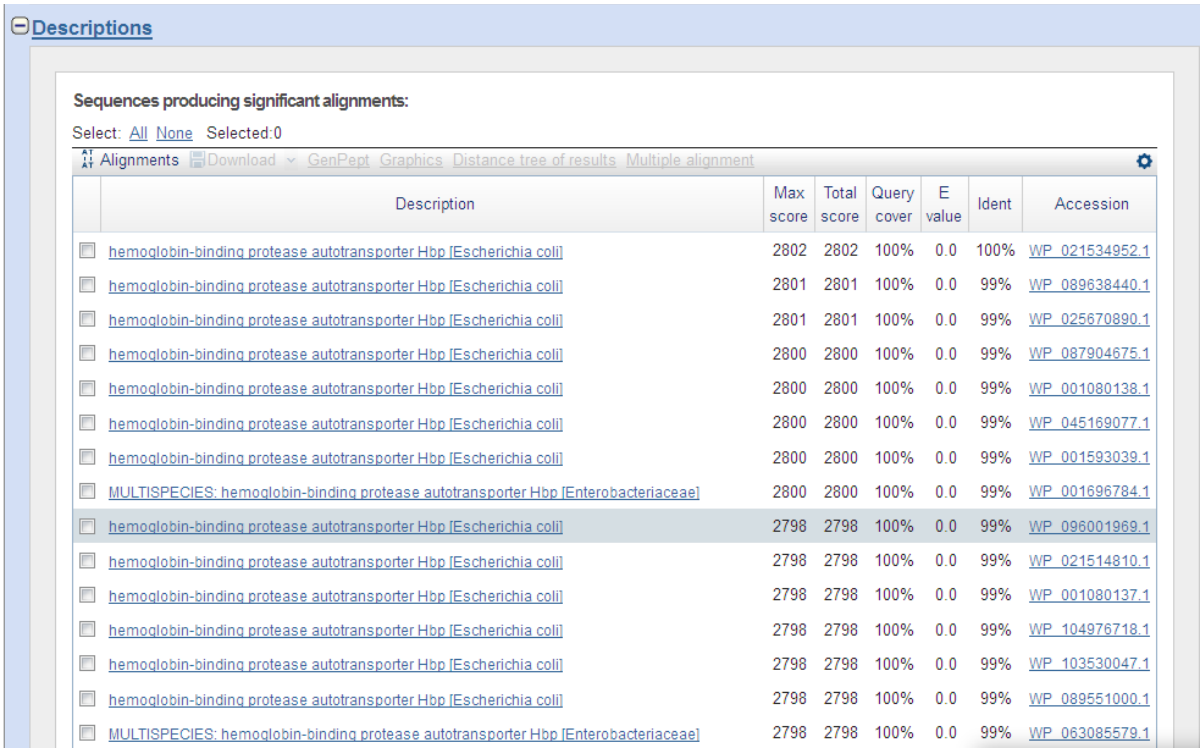


Input page of BLAST



Result of the alignment of the protein





Description	Max score	Total score	Query cover	E value	Ident	Accession
hemoglobin-binding protease autotransporter Hbp [Escherichia coli]	2802	2802	100%	0.0	100%	WP_021534952.1
hemoglobin-binding protease autotransporter Hbp [Escherichia coli]	2801	2801	100%	0.0	99%	WP_089638440.1
hemoglobin-binding protease autotransporter Hbp [Escherichia coli]	2801	2801	100%	0.0	99%	WP_025670890.1
hemoglobin-binding protease autotransporter Hbp [Escherichia coli]	2800	2800	100%	0.0	99%	WP_087904675.1
hemoglobin-binding protease autotransporter Hbp [Escherichia coli]	2800	2800	100%	0.0	99%	WP_001080138.1
hemoglobin-binding protease autotransporter Hbp [Escherichia coli]	2800	2800	100%	0.0	99%	WP_045169077.1
hemoglobin-binding protease autotransporter Hbp [Escherichia coli]	2800	2800	100%	0.0	99%	WP_001593039.1
MULTISPECIES: hemoglobin-binding protease autotransporter Hbp [Enterobacteriaceae]	2800	2800	100%	0.0	99%	WP_001696784.1
hemoglobin-binding protease autotransporter Hbp [Escherichia coli]	2798	2798	100%	0.0	99%	WP_096001969.1
hemoglobin-binding protease autotransporter Hbp [Escherichia coli]	2798	2798	100%	0.0	99%	WP_021514810.1
hemoglobin-binding protease autotransporter Hbp [Escherichia coli]	2798	2798	100%	0.0	99%	WP_001080137.1
hemoglobin-binding protease autotransporter Hbp [Escherichia coli]	2798	2798	100%	0.0	99%	WP_104976718.1
hemoglobin-binding protease autotransporter Hbp [Escherichia coli]	2798	2798	100%	0.0	99%	WP_103530047.1
hemoglobin-binding protease autotransporter Hbp [Escherichia coli]	2798	2798	100%	0.0	99%	WP_089551000.1
MULTISPECIES: hemoglobin-binding protease autotransporter Hbp [Enterobacteriaceae]	2798	2798	100%	0.0	99%	WP_063085579.1

Hemoglobin-binding protease autotransporterHbp [*Escherichia coli*]

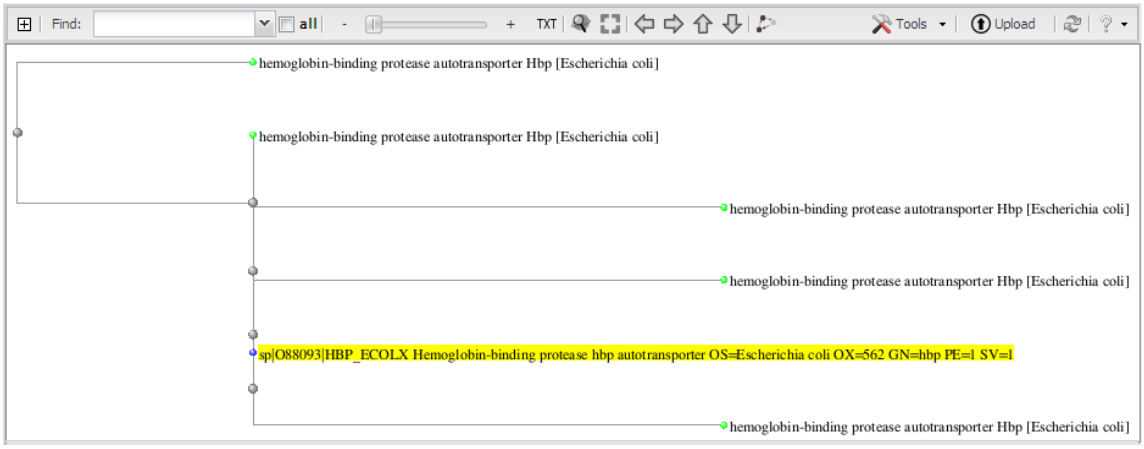
Sequence ID: WP_021531794.1Length: 1377Number of Matches: 1

Alignment statistics for match #1

Score	Expect	Method	Identities	Positives	Gaps
2798 bits(7252)	0.0	Compositional matrix adjust.	1375/1377(99%)	1376/1377(99%)	0/1377(0%)

```
Query  1     MNRIYSLRYSAVARGFIAVSEFARKCVHKSVRRLCFPVLLLIPVLFSAGSLAGTVNNELG  60
             MNRIYSLRYSAVARGFIAVSEFARKCVHKSVRRLCFPVLLLIPVLFSAGSLAGTVNNELG
Sbjct  1     MNRIYSLRYSAVARGFIAVSEFARKCVHKSVRRLCFPVLLLIPVLFSAGSLAGTVNNELG  60
```

```
Query  61    YQLFRDFAENKGMFRPGATNIAIYNKQGEFVGTLDKAAMPDFSAVDSEIGVATLINPQYI  120
             YQLFRDFAENKGMFRPGATNIAIYNKQGEFVGTLDKAAMPDFSAVDSEIGVATLINPQYI
Sbjct  61    YQLFRDFAENKGMFRPGATNIAIYNKQGEFVGTLDKAAMPDFSAVDSEIGVATLINPQYI  120

Query  121   ASVKHNGGYTNVSFGDGENRYNIVDRNNAPSLDFHAPRLDKLVTEVAPTAVTAQGAVAGA  180
             ASVKHNGGYTNVSFGDGENRYNIVDRNNAPSLDFHAPRLDKLVTEVAPTAVTAQGAVAGA
Sbjct  121   ASVKHNGGYTNVSFGDGENRYNIVDRNNAPSLDFHAPRLDKLVTEVAPTAVTAQGAVAGA  180

Query  181   YLDKERYPVFYRLGSGTQYIKDSNGQLTKMGGAYSWLTGGTVGSLSSYQNGEMISTSSGL  240
             YLDKERYPVFYRLGSGTQYIKDSNGQLT+MGGAYSWLTGGTVGSLSSYQNGEMISTSSGL
Sbjct  181   YLDKERYPVFYRLGSGTQYIKDSNGQLTQMGGAYSWLTGGTVGSLSSYQNGEMISTSSGL  240

Query  241   VFDYKLNGAMPIYGEAGDSGSPLFAFDTVQNKWVLVGVLTAGNGAGGRGNNWAVIPLDFI  300
             VFDYKLNGAMPIYGEAGDSGSPLFAFDTVQNKWVLVGVLTAGNGAGGRGNNWAVIPLDFI
Sbjct  241   VFDYKLNGAMPIYGEAGDSGSPLFAFDTVQNKWVLVGVLTAGNGAGGRGNNWAVIPLDFI  300

Query  301   GQKFNEDNDAPVTFRTSEGGALEWSFNSSTGAGALTQGTTTYAMHGQQGNDLNAGKNLIF  360
             GQKFNEDNDAPVTFRTSEGGALEWSFNSSTGAGALTQGTTTYAMHGQQGNDLNAGKNLIF
Sbjct  301   GQKFNEDNDAPVTFRTSEGGALEWSFNSSTGAGALTQGTTTYAMHGQQGNDLNAGKNLIF  360

Query  361   QGQNGQINLKDSVSQGAGSLTFRDNYTVTTSNGSTWTGAGIVVDNGVSVNWQVNGVKGDN  420
             QGQNGQINLKDSVSQGAGSLTFRDNYTVTTSNGSTWTGAGIVVDNGVSVNWQVNGVKGDN
Sbjct  361   QGQNGQINLKDSVSQGAGSLTFRDNYTVTTSNGSTWTGAGIVVDNGVSVNWQVNGVKGDN  420
```



5. Pair wise sequence alignment by using FASTA

FASTA is a DNA and Protein sequence alignment software package. FASTA program was designed for protein sequence similarity searching against protein databases using the FASTA suite of programs. There are several programs in this package that allow the alignment of protein sequences and DNA sequences. FASTA provides a heuristic search with a protein query. FASTX and FASTY translate a DNA query. Optimal searches are available with SSEARCH (local), GGSEARCH (global) and GLSEARCH (global query, local database). The current FASTA package contains programs for protein: protein, DNA: DNA, protein: translated DNA (with frameshifts), and ordered or unordered peptide searches. A major focus of the package is the calculation of accurate similarity statistics, so that biologists can judge whether an alignment is likely to have occurred by chance, or whether it can be used to infer homology.

Steps:

1. Open the FASTA program page from EBI tools (www.ebi.ac.uk/tools/sss/fasta)
2. Retrieve the protein sequence from NCBI database in the FASTA format (www.ncbi.nlm.nih.gov).

3. Select the protein database against which the search has to be carried out.

4. Copy the entire sequence and paste it in the field titled "Enter your input sequence" or upload the file containing sequence.

5. Set the parameters and make sure you have selected the correct FASTA program and database.

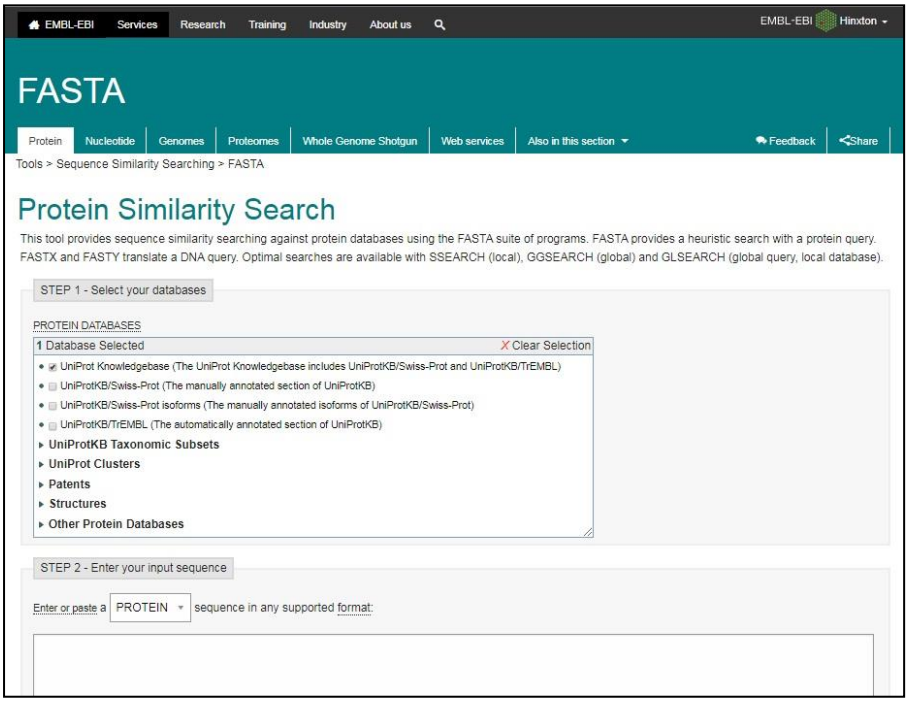
- Matrix: Matrix option is used to set the matrix, which is used for searching the database.
- Gap penalties: it has two options one is Gap opening and Gap extension.
- Default gap opening penalty for proteins is -12 and -16 for DNA. The gap extension penalty is -2 for protein and -4 for DNA.
- Score: Score option gives the maximum number of reported scores in the output file.
- K-tup: Change this value to limit the word length.
- Strand: This option let you chose which strand to search with the respective data band.
- Histogram: Selecting this option to 'yes' will display the search histogram of the expected frequency of chance occurrence of the data base matches found.
- Expectation value upper limit and lower limit: This option is used for score an alignment display. The default values for upper limit are 10.0 for protein search.
- Sequence range: This option allows the user to denote which region within the query sequence should be searched.
- Statistical estimates option is used for statistical calculations.
- Then click the requisite option in different places as per our requirement.

Otherwise leave as such, the programme will take all default option.

7. Submit the job to run.

8. Once your results are computed, they will be presented in the window.

Results:



6. Multiple sequence alignment by using Clustal-W

Multiple sequence alignment (MSA) is a sequence alignment of three or more biological sequences, generally protein, DNA, or RNA. ClustalW is a widely used system for aligning any number of homologous nucleotide or protein sequences. ClustalW is a weighted variant to which access is provided by a large number of web portals including GenomeNet, EBI, and EMBNet. ClustalW helps in improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. ClustalW is used extensively for phylogenetic tree construction, which can be seen via. viewing cladogram and phylogram. From the resulting output, sequence homology can be inferred and phylogenetic analysis can be conducted to assess the sequences' shared evolutionary origins.

Steps:

1. Open the ClustalW program page from GenomeNet tool.
2. Retrieve more than 2 sequences (either DNA or Protein) from NCBI database (www.ncbi.nlm.nih.gov) in FASTA format.
3. Paste the retrieved sequences onto program query box of ClustalW with labels or upload the file containing sequence.
4. Click the label Protein or DNA according to the sequence of interest.
5. Then click the requisite option in different places as per our requirement. Otherwise leave as such, the programme will take all default option.
6. Run the ClustalW.

Results:

Input File

1: FJ240161. Reports Populusbalsamife...[gi:226232336]

- Next sequence

```
>gi|226232336|gb|FJ240161.1| Populusbalsamifera isolate Nom02 kunitz trypsin
inhibitor 4 (KTI4) gene, partial cds
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TTTATTTTGAGAATAATAGAGGTAGAATTCTGTCTCTTAGTGATAAACTGTC
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2: FJ240160. Reports Populusbalsamife...[gi:226232334]

- Previous sequence
- Next sequence

```
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TAAAACGTGCCCGGATGATGTTATTCAATACTCGTTGGACCAGTTACAAGGTCTTCCAGTTACCTTCTCA
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3: FJ240164. Reports Populusbalsamife...[gi:226232342]

- Previous sequence
- Next sequence

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4: FJ240163. Reports Populusbalsamife...[gi:226232340]

- Previous sequence
- Next sequence

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5: FJ240162. Reports Populusbalsamife...[gi:226232338]

- Previous sequence

>gi|226232338|gb|FJ240162.1| Populusbalsamifera isolate Nom02 kunitz trypsin inhibitor 4 (KTI4) gene, partial cds
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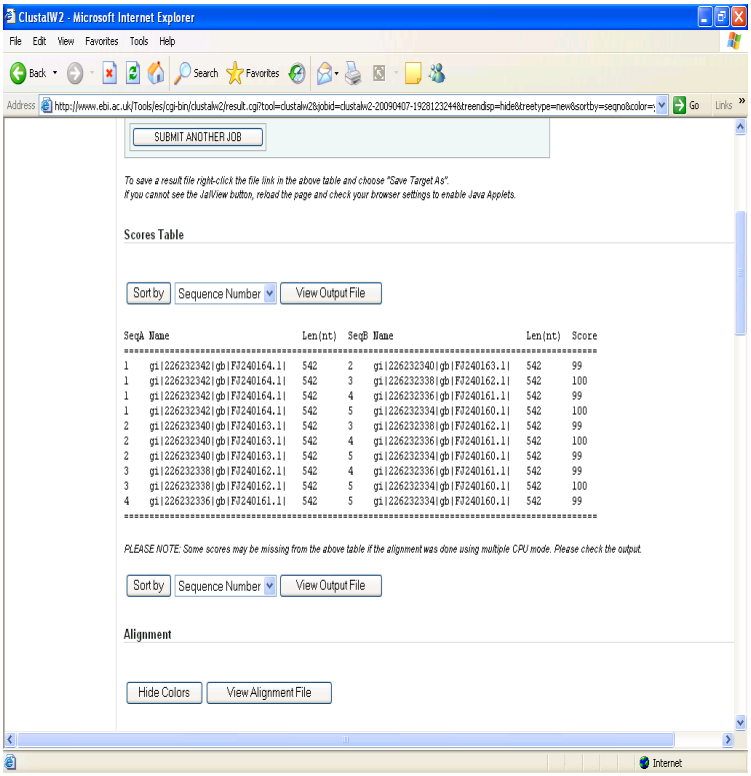
Output File


ClustalW2 - Microsoft Internet Explorer
File Edit View Favorites Tools Help
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SUBMIT ANOTHER JOB
To save a result file right-click the file link in the above table and choose "Save Target As".
If you cannot see the JalView button, reload the page and check your browser settings to enable Java Applets.
Scores Table
Sort by Sequence Number View Output File
SeqA Name Len(nt) SeqB Name Len(nt) Score
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1 gi|226232342|gb|FJ240164.1| 542 3 gi|226232338|gb|FJ240162.1| 542 100
1 gi|226232342|gb|FJ240164.1| 542 4 gi|226232336|gb|FJ240161.1| 542 99
1 gi|226232342|gb|FJ240164.1| 542 5 gi|226232334|gb|FJ240160.1| 542 100
2 gi|226232340|gb|FJ240163.1| 542 3 gi|226232338|gb|FJ240162.1| 542 99
2 gi|226232340|gb|FJ240163.1| 542 4 gi|226232336|gb|FJ240161.1| 542 100
2 gi|226232340|gb|FJ240163.1| 542 5 gi|226232334|gb|FJ240160.1| 542 99
3 gi|226232338|gb|FJ240162.1| 542 4 gi|226232336|gb|FJ240161.1| 542 99
3 gi|226232338|gb|FJ240162.1| 542 5 gi|226232334|gb|FJ240160.1| 542 100
4 gi|226232336|gb|FJ240161.1| 542 5 gi|226232334|gb|FJ240160.1| 542 99
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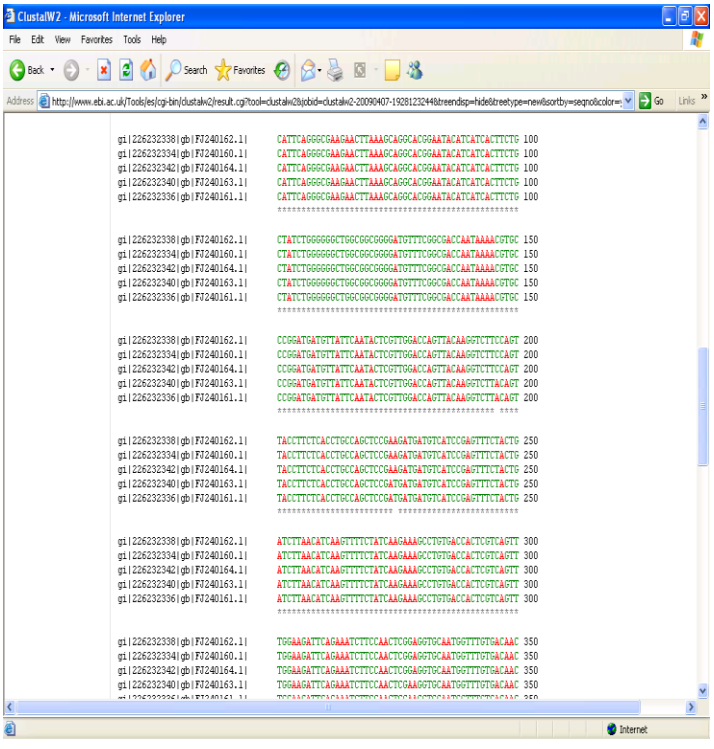
Sort by Sequence Number View Output File
Alignment
Hide Colors View Alignment File
Internet


ClustalW2 - Microsoft Internet Explorer
File Edit View Favorites Tools Help
Back Search Favorites
Address http://www.ebi.ac.uk/Tools/es/cgi-bin/clustalw2/result.cgi?tool=clustalw2&jobid=clustalw2-20090407-192812324&treendisp=hide&treetype=new&sortby=seqno&color=
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Internet

7. Construction of Phylogenetic Tree

Phylogenetic methods can be used for many purposes, including analysis of morphological and several kinds of molecular data. The analysis of DNA and protein sequences involves.

- Comparisons of more than two sequences
- Analysis of gene families, including functional predictions
- Estimation of evolutionary relationship among organisms

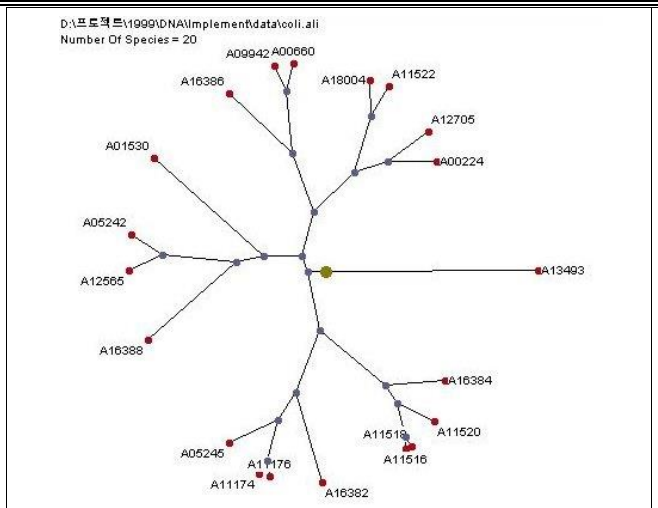
The basic concepts of phylogenetic analysis are quite easy to understand, but understanding what the results of the analysis mean, and avoiding errors of analysis can be quite difficult. The basic model of phylogenetic analysis such UPGMA Method, Maximum Parsimony, Maximum Likelihood. Nearly all methods of phylogenetic analysis share a number of fundamental assumptions. These include: Homologous sequences are in a multiple sequence alignment.

Note that homology is an a priori assumption of most phylogenetic methods. If homology is uncertain, then the analytical results should be interpreted with great caution. The alignment is also referred to as a data matrix.

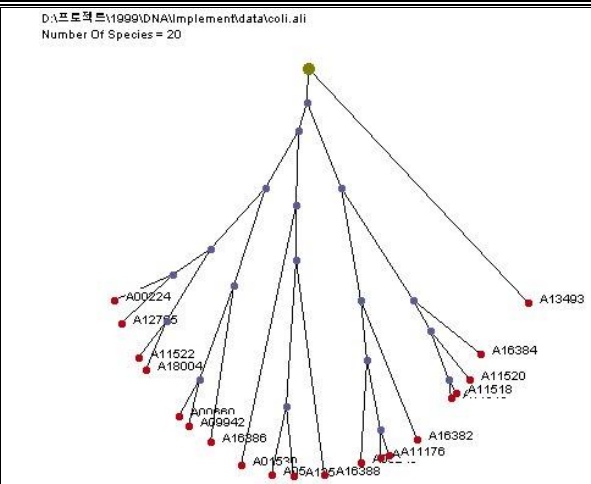
Each column in the alignment is referred to as a character. The specific residue (nucleotide or amino acid) present in a given sequence is referred to as the character state. They are assumed to have been derived from a single common ancestor (this statement is actually redundant; by definition homologous sequences must be derived from a common ancestor). In most cases ancestral sequences are not known, and the ancestral states must be inferred. The ancestral sequences are assumed to have undergone mutation. Modelling mutation accurately is one of the challenges of phylogenetic analysis.

They are assumed to be related by a dichotomously branching tree. A priori assumption include (but are not necessarily limited to) the accuracy of sequence. The accuracy of the sequence is based on the sequence itself is correct and was determined from the correct organism. Various softwares are available for phylogenetic analysis. One among is PHYLODRAW software. The general description of Phylodraw is given below:

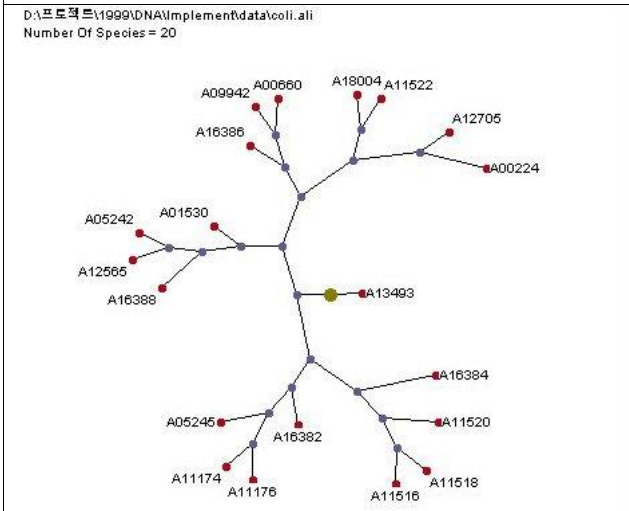
PhyloDraw is a drawing tool for creating phylogenetic trees. PhyloDraw supports various kinds of multialignment programs (Dialign2, ClustalW, Phylip format, and pairwise distance matrix) and visualizes various kinds of tree diagrams, e.g. rectangular cladogram, slanted cladogram, phylogram, free tree, and radial tree. With PhyloDraw users can manipulate the shape of a phylogenetic tree easily and interactively by using several control parameters. This program can export the final tree layout to BMP (bitmap image format) and Post script.



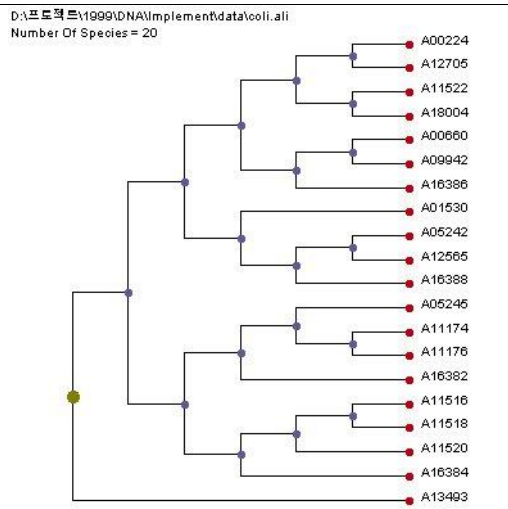
Free tree



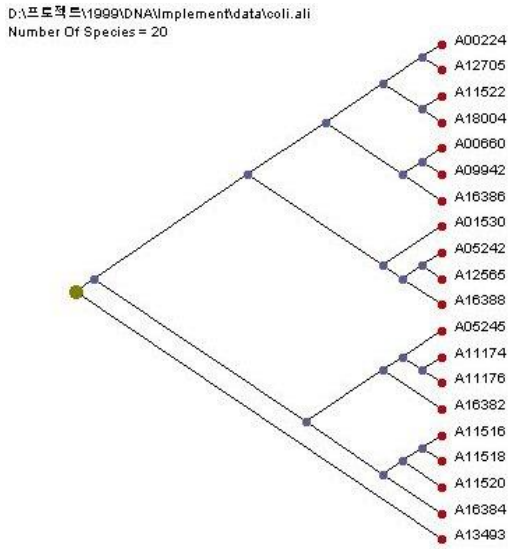
Rooted Tree



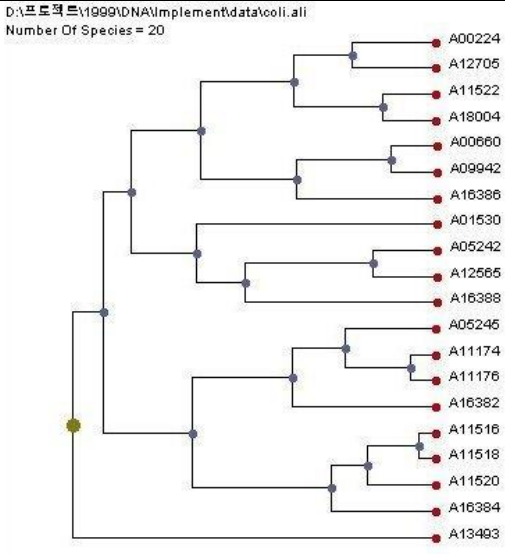
Radial Tree



Rectangle Cladogram



Slanted Cladogram



Phylogram

8. Retrieving PBD sequence data &3D structure of protein by using Rasmol

Rasmol is a molecular graphics program intended for the visualization of proteins nucleic acids and small molecules. The program is aimed at display texting and generation of publication quality images. The program leads in a molecule co-ordinate file and interactively displays the molecule on the screen in a variety of color schemes and molecule representations. Currently available representations include depth- wire frames, space filling spheres, ball and stick, solid and strand bio-molecular ribbons, atom labels and dot surfaces. Up to 5 may be loaded and displayed at once. Any one (or) all of the molecules may be rotated and translated.

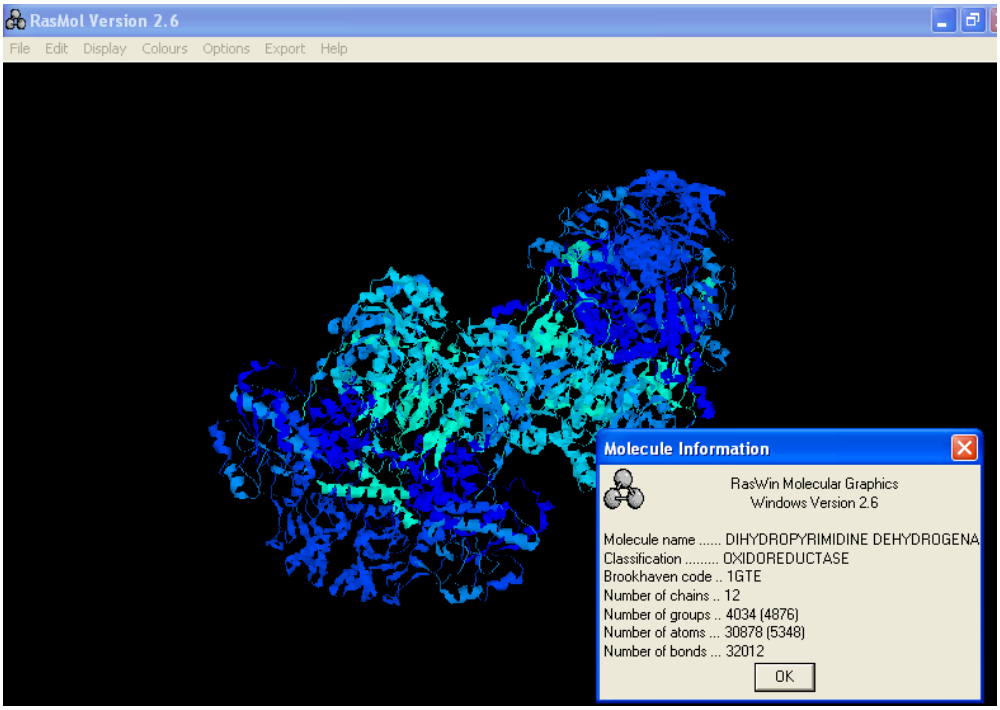
Procedure

1. The Rasmol 2.6 software was downloaded from www.rasmol.org.
2. To view the structure of the PDB file, file menu in the Rasmol window was selected.
3. In the submenu, open option was clicked and saved and PDB file was selected to be opened.
4. The 3D structure of the PDB data was displayed in the Rasmol window.

Result

The converted PDB format of protein sequence was visualized in 3D structure using Rasmol.

Thymidylate synthase



SummaryActivities

- *In-silico* tools like sequence alignment, pair wise comparison, multiple sequence alignment of both nucleotide and protein were performed.
- Phylogenic tree were constructed with the microbial sequences.
- Protein structure predictions were analysed.

References:

Ekins, S., Mestres, J., & Testa, B. (2007). In silico pharmacology for drug discovery: methods for virtual ligand screening and profiling. *British journal of pharmacology*, *152*(1), 9–20. https://doi.org/10.1038/sj.bjp.0707305

Kinnings, S.L., Liu, N., Buchmeier, N., Tonge, P.J., Xie, L., et al. (2009). Drug Discovery Using Chemical Systems Biology: Repositioning the Safe Medicine Comtan to Treat Multi-Drug and Extensively Drug Resistant Tuberculosis. *PLoSComput Biol*, 5(7): e1000423. https://doi.org/10.1371/journal.pcbi.1000423

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Chandra Sekhar Mukhopadhyay, Ratan Kumar Choudhary, Mir Asif Iquebal (2017)Basic Applied Bioinformatics. Wiley publication.

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