LECTURE NOTE _ (6th Sem) DOWN STREAM PROCESSING

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TOPIC : DISTILLATION

Distillation

- Distillation is the process of separating components of a mixture based on different boiling points.
- Examples of uses of distillation include purification of alcohol, desalination, crude oil refining, and making liquefied gases from air.
- Humans have been using distillation since at least 3000 BC in the Indus valley.

Distillation Definition

Distillation is a widely used method for separating mixtures based on differences in the conditions required to change the phase of components of the mixture. To separate a mixture of liquids, the liquid can be heated to force components, which have different boiling points, into the gas phase. The gas is then condensed back into liquid form and collected. Repeating the process on the collected liquid to improve the purity of the product is called double distillation. Although the term is most commonly applied to liquids, the reverse process can be used to separate gases by liquefying components using changes in temperature and/or pressure.

A plant that performs distillation is called a *distillery*. The apparatus used to perform distillation is called a *still*.

History

The earliest known evidence of distillation comes from a terracotta distillation apparatus dating to 3000 BC in the Indus valley of Pakistan. Distillation was known to be used by the Babylonians of Mesopotamia. Initially, distillation is believed to have been used to make perfumes. Distillation of beverages occurred much later. The Arab chemist Al-Kindi distilled alcohol in 9th century Irag. Distillation of alcoholic beverages appears common in Italy and China starting in the 12th century.

Uses of Distillation

Distillation is used for many commercial processes, such as the production of gasoline, distilled water, xylene, alcohol, paraffin, kerosene, and many other liquids. Gas may be liquefied and separate. For example: nitrogen, oxygen, and argon are distilled from air.

Types of Distillation

Types of distillation include simple distillation, fractional distillation (different volatile 'fractions' are collected as they are produced), and destructive distillation (usually, a material is heated so that it decomposes into compounds for collection).

Simple Distillation

Simple distillation may be used when the boiling points of two liquids are significantly different from each other or to separate liquids from solids or nonvolatile components. In simple distillation, a mixture is heated to change the most volatile component from a liquid into vapor. The vapor rises and passes into a condenser. Usually, the condenser is cooled (e.g., by running cold water around it) to promote condensation of the vapor, which is collected.

Steam Distillation

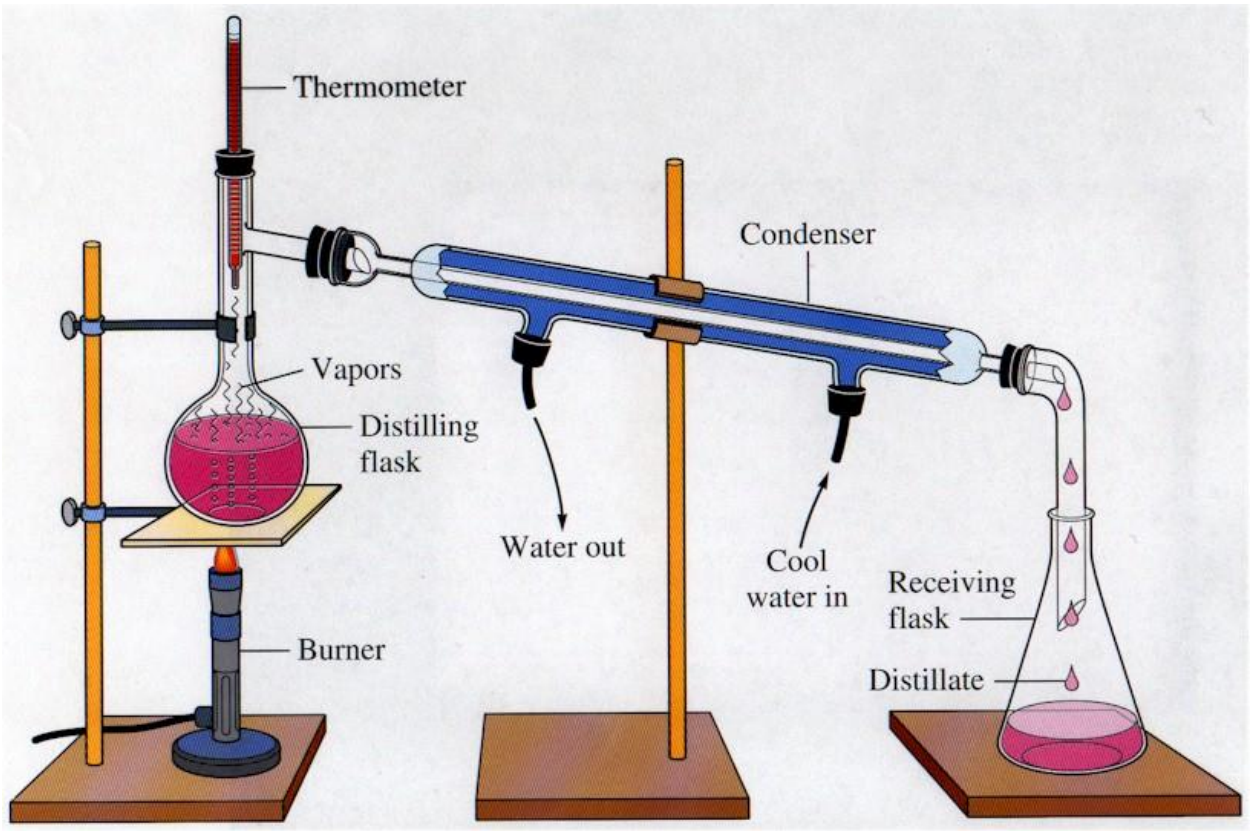
Steam distillation is used to separate heat-sensitive components. Steam is added to the mixture, causing some of it to vaporize. This vapor is cooled and condensed into two liquid fractions. Sometimes the fractions are collected separately, or they may have different density values, so they separate on their own. An example is steam distillation of flowers to yield essential oil and a water-based distillate.

Fractional Distillation

Fractional distillation is used when the boiling points of the components of a mixture are close to each other, as determined using Raoult's law. A fractionating column is used to separate the components used a series of distillations called rectification. In fractional distillation, a mixture is heated so vapor rises and enters the fractionating column. As the vapor cools, it condenses on the packing material of the column. The heat of rising vapor causes this liquid to vaporize again, moving it along the column and eventually yielding a higher purity sample of the more volatile component of the mixture.

Vacuum Distillation

Vacuum distillation is used to separate components that have high boiling points. Lowering the pressure of the apparatus also lowers boiling points. Otherwise, the process is similar to other forms of distillation. Vacuum distillation is particularly useful when the normal boiling point exceeds the decomposition temperature of a compound.



Many of the hazards associated with the distillation process have been discussed in previous sections (glassware, flowing water, heating devices). However, the importance of the procedure is such that it is advisable to collect the warnings in this one place to make sure nothing goes unnoticed.

• The apparatus is usually made of glass and therefore subject to breakage.• All components of the distillation apparatus should be secured to a stable stand or rack to prevent it from falling over.• All the glassware, particularly the part to be heated, should be checked for cracks prior to use.• Connections between the glass parts may involve rubber or cork stoppers but in more modern apparatus standard taper connections are used.• If stoppers are used, it must be known that the hot vapors will not react with the rubber or cork and thus contaminate the products.• If standard taper connections are used, any lubricant used to make tight seals must also not react, melt or evaporate and contaminate the product.

• The condenser must generally be connected to a source of running water to provide cooling for the vapors.• The proper method is to connect the input hose to the condenser at the end furthest from the heated flask and the outflow hose nearest the heated flask.• This prevents the hottest vapors from contacting the coldest water and creating a large thermal shock to the glassware.• As mentioned in the section on flowing water, the hoses must be connected tightly enough to the condenser that they will not come loose if the water pressure should increase during the experiment.• Usually this means that something like copper wire is twisted around

the tubing at the joint to prevent it from coming away. The flow of water must be sufficient to accomplish condensation without being so fast as to cause undue hose pressure or splashing of outflow water, remembering that flow rates can change during the day after they have initially been set.

 The distillation flask should preferably be a round-bottomed one rather than a flat-bottomed one for smoothness of boiling. The flask should never be more than half-filled with the liquid mixture to be distilled. Greater filling leads to bubbles and sometimes foaming that is constricted in the narrowing part of the flask and gets out of control.

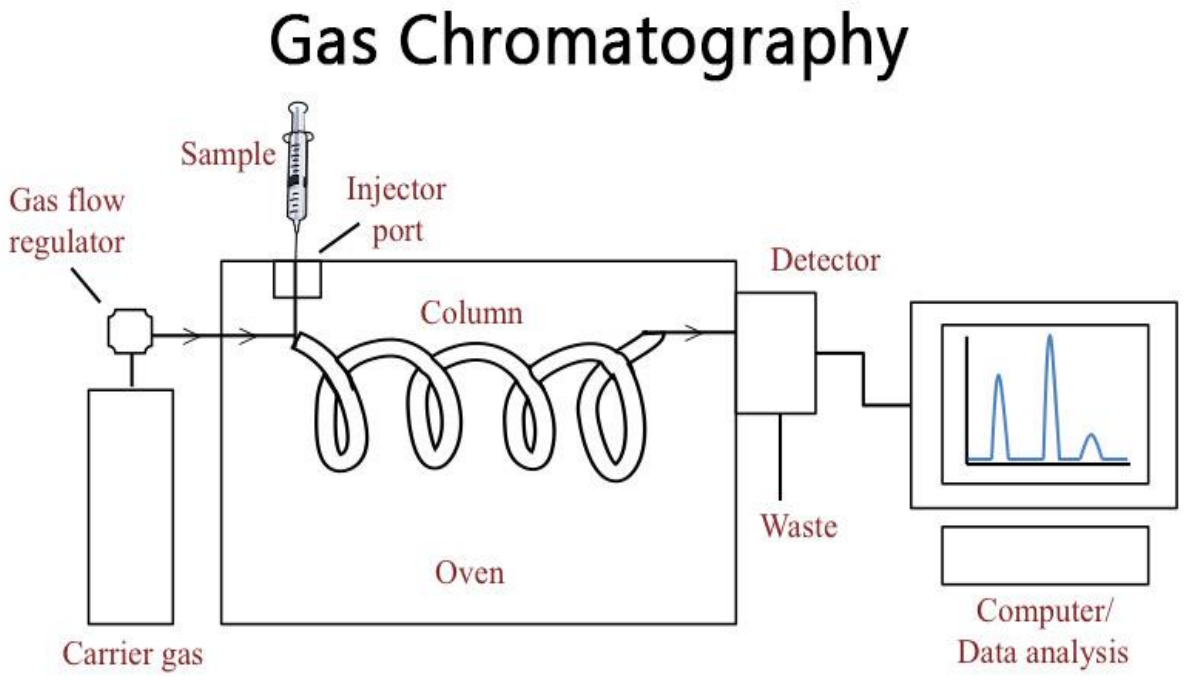
 To make boiling smoothest, boiling chips or tubes should be added to the liquid in the distillation flask before heating has begun. It is very important not to add chips or tubes to heated liquid as it may suddenly begin to boil and eject hot liquid out onto the operator. The chips are generally made of sharp pieces of broken ceramic or hard plastic. Tubes are usually of the capillary type with both ends open.

 Heating the distillation flask requires care. The liquids being distilled are often flammable so that flame is not the preferred heat source. Heating mantles or sand baths are good sources of heat to conform to the round-bottomed flasks. Care must be taken not to let any vapors near the control switches that may spark when opening and closing. Doing the distillation inside a hood is a good practice.

One last word of caution about the apparatus is in order. There have been cases where the operators decided to make the connection between the condenser and the receiving vessel a tight one using a stopper or standard taper connection. This must NOT be done as it creates a completely sealed system. When the distillation flask is heated and vapors begin to rise they will expand and create a pressure in such a sealed system that will inevitably blow the joints apart. This generally causes vapors to escape into the surrounding room (or hopefully hood) if not the glassware to be broken. Always allow for a pressure relief opening in the distillation system between the condenser and the receiving vessel. Note in the diagram above the glass connector where the drops are falling is NOT sealed but is only loosely in the receiving flask.

What is gas chromatography?

- Gas chromatography differs from other forms of **chromatography** in that the mobile phase is a gas and the components are separated as vapors.
- It is thus used to separate and detect small molecular weight compounds in the gas phase.
- The sample is either a gas or a liquid that is vaporized in the injection port. The mobile phase for gas chromatography is a carrier gas, typically helium because of its low molecular weight and being chemically inert.
- The pressure is applied and the mobile phase moves the analyte through the column. The separation is accomplished using a column coated with a stationary phase.



Principle of Gas chromatography (how does gas chromatography work)

The equilibrium for gas chromatography is partitioning, and the components of the sample will partition (i.e. distribute) between the two phases: the stationary phase and the mobile phase.

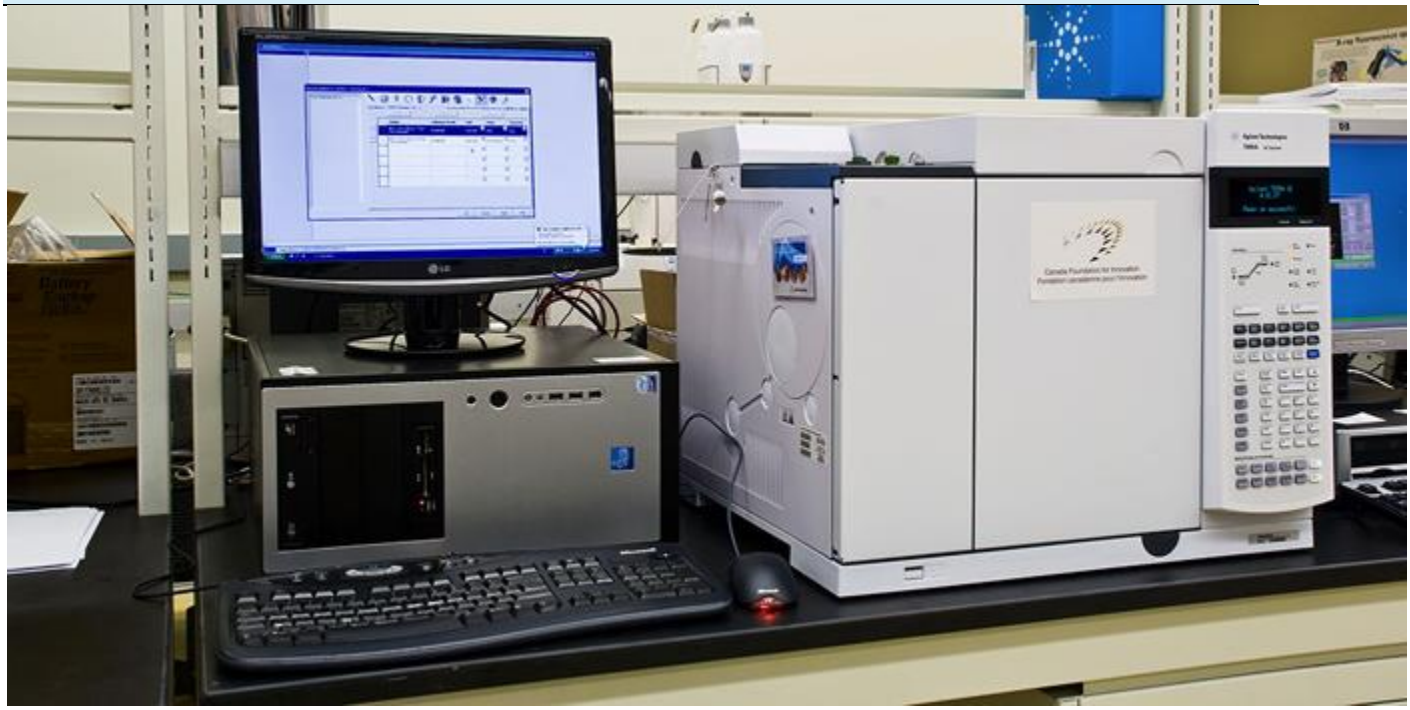
Compounds that have a greater affinity for the stationary phase spend more time in the column and thus elute later and have a longer **retention time (Rt)** than samples that have a higher affinity for the mobile phase.

Affinity for the stationary phase is driven mainly by intermolecular interactions and the polarity of the stationary phase can be chosen to maximize interactions and thus the separation.

Ideal peaks are Gaussian distributions and symmetrical, because of the random nature of the analyte interactions with the column.

- The separation is hence accomplished by partitioning the sample between the gas and a thin layer of a nonvolatile liquid held on a solid support.
- A sample containing the solutes is injected into a heated block where it is immediately vaporized and swept as a plug of vapor by the carrier gas stream into the column inlet.
- The solutes are adsorbed by the stationary phase and then desorbed by a fresh carrier gas.
- The process is repeated in each plate as the sample is moved toward the outlet.
- Each solute will travel at its own rate through the column.
- Their bands will separate into distinct zones depending on the partition coefficients, and band spreading.
- The solutes are eluted one after another in the increasing order of their kd, and enter into a detector attached to the exit end of the column.
- Here they register a series of signals resulting from concentration changes and rates of elution on the recorder as a plot of time versus the composition of carrier gas stream.
- The appearance time, height, width, and area of these peaks can be measured to yield quantitative data.

Parts of Gas chromatography



Gas chromatography is mainly composed of the following parts:

1. **Carrier gas in a high-pressure cylinder with attendant pressure regulators and flow meters**

- Helium, N_2, H, Argon are used as carrier gases.
- Helium is preferred for thermal conductivity detectors because of its high thermal conductivity relative to that of most organic vapors.
- N_2 is preferable when a large consumption of carrier gas is employed.
- Carrier gas from the tank passes through a toggle valve, a flow meter, (1-1000 ml/min), capillary restrictors, and a pressure gauge (1-4 atm).
- Flow rate is adjusted by means of a needle valve mounted on the base of the flow meter and controlled by capillary restrictors.
- The operating efficiency of the gas chromatograph is directly dependant on the maintenance of constant gas flow.

2. **Sample injection system**

- Liquid samples are injected by a microsyringe with a needle inserted through a self-scaling, silicon-rubber septum into a heated metal block by a resistance heater.
- Gaseous samples are injected by a gas-tight syringe or through a by-pass loop and valves.
- Typical sample volumes range from 0.1 to 0.2 ml.

3. **The separation column**

- The heart of the gas chromatography is the column which is made of metals bent in U shape or coiled into an open spiral or a flat pancake shape.
- Copper is useful up to 250^0
- Swege lock fittings make column insertion easy.
- Several sizes of columns are used depending upon the requirements.

4. **Liquid phases**

- An infinite variety of liquid phases are available limited only by their volatility, thermal stability and ability to wet the support.
- No single phase will serve for all separation problems at all temperatures.

Non-Polar – Parafin, squalane, silicone greases, apiezon L, silicone gum rubber. These materials separate the components in order of their boiling points.

Intermediate Polarity – These materials contain a polar or polarizable group on a long non-polar skeleton which can dissolve both polar and non-polar solutes. For example. diethyl hexyl phthalate is used for the separation of high boiling alcohols.

Polar – Carbowaxes – Liquid phases with a large proportion of polar groups. Separation of polar and non-polar substances.

Hydrogen bonding – Polar liquid phases with high hydrogen bonding e.g. Glycol.

Specific purpose phases – Relying on a chemical reaction with solute to achieve separations. e.g AgNO3 in glycol separates unsaturated hydrocarbons.

5. **Supports**

- The structure and surface characteristics of the support materials are important parameters, which determine the efficiency of the support and the degree of separation respectively.
- The support should be inert but capable of immobilizing a large volume of liquid phase as a thin film over its surface.
- The surface area should be large to ensure the rapid attainment of equilibrium between stationary and mobile phases.
- Support should be strong enough to resist breakdown in handling and be capable of packed into a uniform bed.
- Diatomaceous earth, kieselguhr treated with Na 2CO 3 for 900^0 C causes the particle fusion into coarser aggregates.
- Glass beads with a low surface area and low porosity can be used to coat up to 3% stationary phases.
- Porous polymer beads differing in the degree of cross-linking of styrene with alkyl-vinyl benzene are also used which are stable up to 250^0

6. **Detector**

- Detectors sense the arrival of the separated components and provide a signal.
- These are either concentration-dependent or mass dependant.
- The detector should be close to the column exit and the correct temperature to prevent decomposition.

7. **Recorder**

- The recorder should be generally 10 mv (full scale) fitted with a fast response pen (1 sec or less). The recorder should be connected with a series of good quality resistances connected across the input to attenuate the large signals.
- An integrator may be a good addition.

The procedure of Gas Chromatography

Step 1: Sample Injection and Vapourization

1. A small amount of liquid sample to be analyzed is drawn up into a syringe.
2. The syringe needle is positioned in the hot injection port of the gas chromatograph and the sample is injected quickly.
3. The injection of the sample is considered to be a "point" in time, that is, it is assumed that the entire sample enters the gas chromatograph at the same time, so the sample must be injected quickly.
4. The temperature is set to be higher than the boiling points of the components of the mixture so that the components will vaporize.
5. The vaporized components then mix with the inert gas mobile phase to be carried to the gas chromatography column to be separated.

Step 2: Separation in the Column

- Components in the mixture are separated based on their abilities to adsorb on or bind to, the stationary phase.
- A component that adsorbs most strongly to the stationary phase will spend the most time in the column (will be retained in the column for the longest time) and will, therefore, have the longest retention time (Rt). It will emerge from the gas chromatograph last.
- A component that adsorbs the least strongly to the stationary phase will spend the least time in the column (will be retained in the column for the shortest time) and will, therefore, have the shortest retention time (Rt). It will emerge from the gas chromatograph first.
- If we consider a 2 component mixture in which component A is more polar than component B then:

1. component A will have a **longer retention time** in a polar column than component B
2. component A will have a **shorter retention time** in a non-polar column than component B

Step 3: Detecting and Recording Results

1. The components of the mixture reach the detector at different times due to differences in the time they are retained in the column.
2. The component that is retained the shortest time in the column is detected first. The component that is retained the longest time in the column is detected last.
3. The detector sends a signal to the chart recorder which results in a peak on the chart paper. The component that is detected first is recorded first. The component that is detected last is recorded last.

Applications

- GC analysis is used to calculate the content of a chemical product, for example in assuring the quality of products in the chemical industry; or measuring toxic substances in soil, air or water.
- Gas chromatography is used in the analysis of:

(a) air-borne pollutants
(b) performance-enhancing drugs in athlete's urine samples
(c) oil spills
(d) essential oils in perfume preparation

- GC is very accurate if used properly and can measure picomoles of a substance in a 1 ml liquid sample, or parts-per-billion concentrations in gaseous samples.
- Gas Chromatography is used extensively in forensic science. Disciplines as diverse as solid drug dose (pre-consumption form) identification and quantification, arson investigation, paint chip analysis, and toxicology cases, employ GC to identify and quantify various biological specimens and crime-scene evidence.

Advantages

- The use of longer columns and higher velocity of carrier gas permits the fast separation in a matter of a few minutes.
- Higher working temperatures up to 5000C and the possibility of converting any material into a volatile component make gas chromatography one of the most versatile techniques.
- GC is popular for environmental monitoring and industrial applications because it is very reliable and can be run nearly continuously.
- GC is typically used in applications where small, volatile molecules are detected and with non-aqueous solutions.
- GC is favored for non-polar molecules.

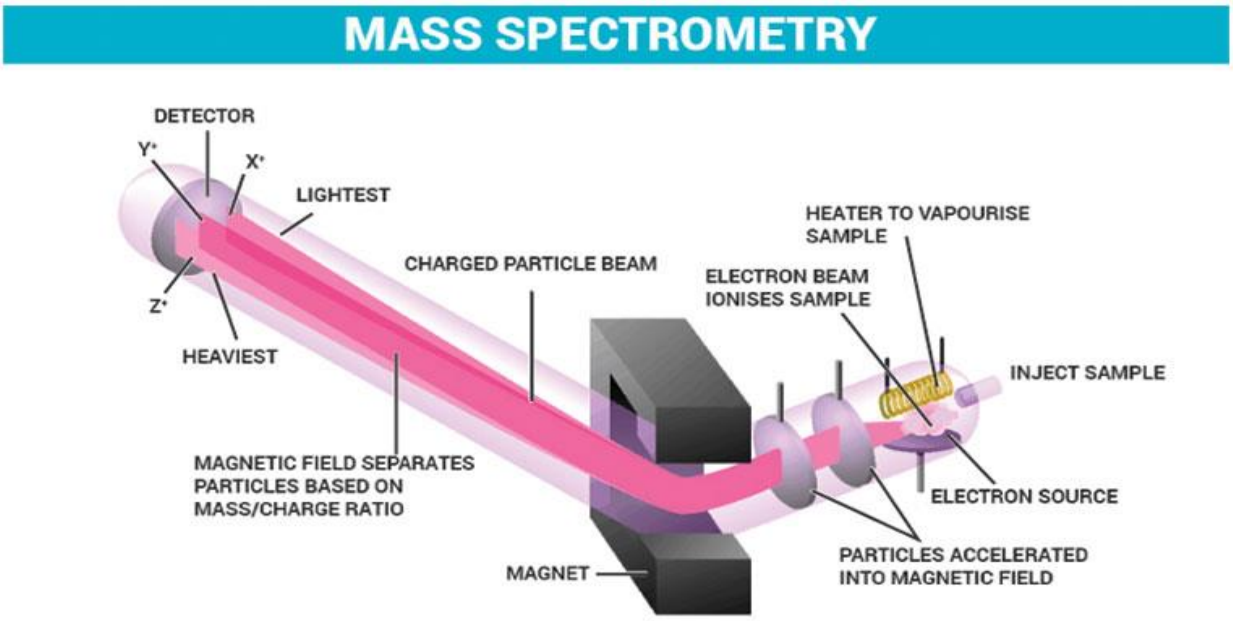
Limitations

- Compound to be analyzed should be stable under GC operation conditions.
- They should have a vapor pressure significantly greater than zero.
- Typically, the compounds analyzed are less than 1,000 Da, because it is difficult to vaporize larger compounds.
- The samples are also required to be salt-free; they should not contain ions.
- Very minute amounts of a substance can be measured, but it is often required that the sample must be measured in comparison to a sample containing the pure, suspected substance known as a reference standard.

Mass Spectrometry (MS)- Principle, Working, Instrumentation, Steps, Applications

- Mass Spectrometry (MS) is an analytical chemistry technique that helps identify the amount and type of chemicals present in a sample by measuring the mass-to-charge ratio and abundance of gas-phase ions.
- In this instrumental technique, sample is converted to rapidly moving positive ions by electron bombardment and charged particles are separated according to their masses.
- Mass spectrum is a plot of relative abundance against the ratio of mass/charge (m/e).
- These spectra are used to determine the elemental or isotopic signature of a sample, the masses of particles and of molecules, and to elucidate the chemical structures of molecules and other chemical compounds.

Principle of Mass Spectrometry (MS)

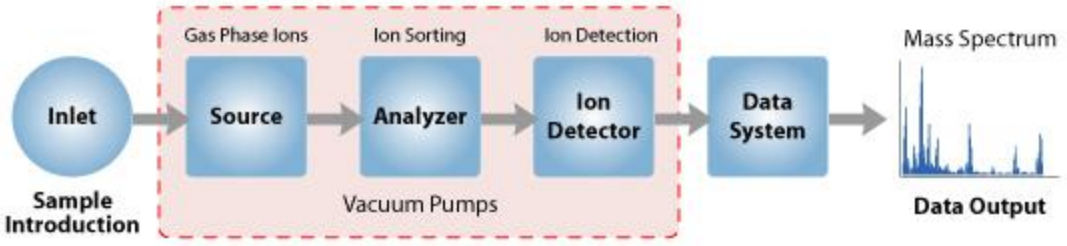


1. In this technique, molecules are bombarded with a beam of energetic electrons.
2. The molecules are ionized and broken up into many fragments, some of which are positive ions. Each kind of ion has a particular ratio of mass to charge, i.e. m/e ratio (value).
3. For most ions, the charge is one and thus, m/e ratio is simply the molecular mass of the ion.
4. The ions pass through magnetic and electric fields to reach detector where they are detected and signals are recorded to give a mass spectra.

Working of Mass Spectrometry (MS)

- In a typical procedure, a sample, which may be solid, liquid, or gas, is ionized, for example by bombarding it with electrons.
- This may cause some of the sample's molecules to break into charged fragments. These ions are then separated according to their mass-to-charge ratio, typically by accelerating them and subjecting them to an electric or magnetic field:
- Ions of the same mass-to-charge ratio will undergo the same amount of deflection.
- The ions are detected by a mechanism capable of detecting charged particles, such as an electron multiplier. Results are displayed as spectra of the relative abundance of detected ions as a function of the mass-to-charge ratio.
- The atoms or molecules in the sample can be identified by correlating known masses (e.g. an entire molecule) to the identified masses or through a characteristic fragmentation pattern.

Instrumentation and Steps of Mass Spectrometry (MS)



A. Sample Inlet

- Sample stored in large reservoir from which molecules reaches ionization chamber at low pressure in steady stream by a pinhole called "Molecular leak".

B. Ionization

- Atoms are ionized by knocking one or more electrons off to give positive ions by bombardment with a stream of electrons. Most of the positive ions formed will carry charge of +1.
- Ionization can be achieved by :
  - Electron Ionization (EI-MS)
  - Chemical Ionization (CI-MS)
  - Desorption Technique (FAB)

C. Acceleration

- Ions are accelerated so that they all have same kinetic energy.
- Positive ions pass through 3 slits with voltage in decreasing order.
- Middle slit carries intermediate and finals at zero volts.

D. Deflection

- Ions are deflected by a magnetic field due to difference in their masses.
- The lighter the mass, more they are deflected.
- It also depends upon the no. of +ve charge an ion is carrying; the more +ve charge, more it will be deflected.

E. Detection

- The beam of ions passing through the mass analyzer is detected by detector on the basis of m/e ratio.
- When an ion hit the metal box, charge is neutralized by an electron jumping from metal on to the ion.
- Types of analyzers:
  - Magnetic sector mass analysers
  - Double focussing analysers
  - Quadrupole mass analysers
  - Time of Flight analysers (TOF)
  - Ion trap analyser

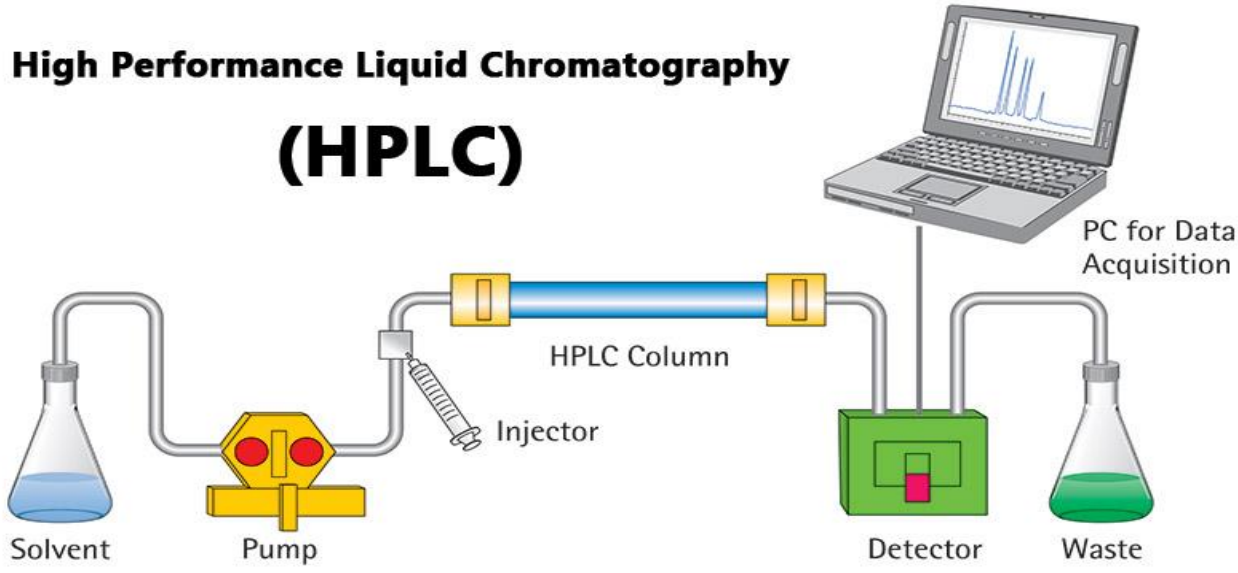
- Ion cyclotron analyser

Applications of Mass Spectrometry (MS)

- Environmental monitoring and analysis (soil, water and air pollutants, water quality, etc.)
- Geochemistry – age determination, soil and rock composition, oil and gas surveying
- Chemical and Petrochemical industry – Quality control
- Identify structures of biomolecules, such as carbohydrates, nucleic acids
- Sequence biopolymers such as proteins and oligosaccharides
- Determination of molecular mass of peptides, proteins, and oligonucleotides.
- Monitoring gases in patients breath during surgery.
- Identification of drugs abuse and metabolites of drugs of abuse in blood, urine, and saliva.
- Analyses of aerosol particles.
- Determination of pesticides residues in food

High-Performance Liquid Chromatography (HPLC)

- High performance liquid chromatography or commonly known as HPLC is an analytical technique used to separate, identify or quantify each component in a mixture.
- The mixture is separated using the basic principle of column **chromatography** and then identified and quantified by spectroscopy.
- In the 1960s the column chromatography LC with its low-pressure suitable glass columns was further developed to the HPLC with its high-pressure adapted metal columns.
- HPLC is thus basically a highly improved form of column liquid chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres.

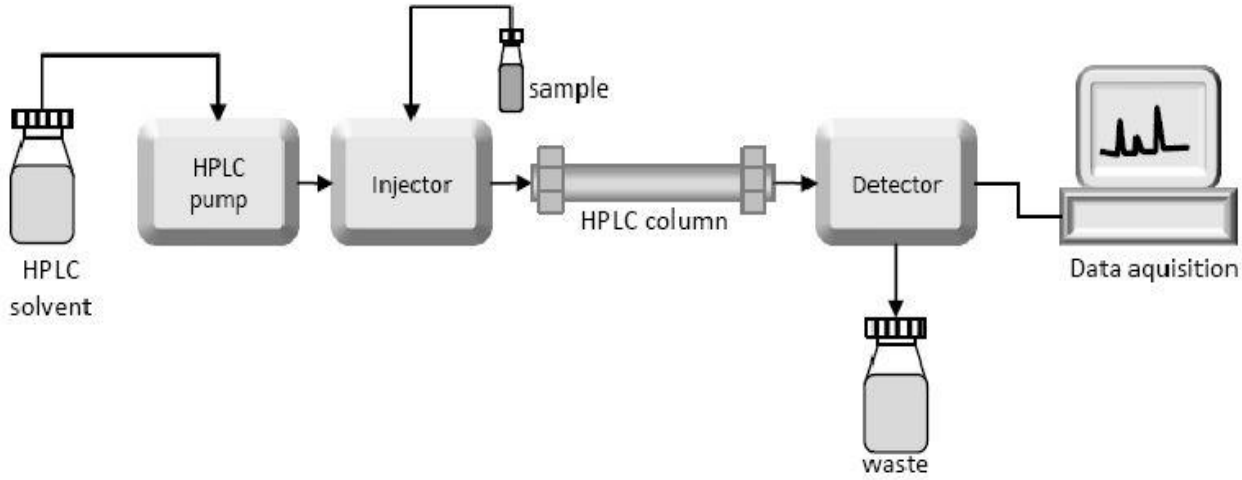


Principle of High-Performance Liquid Chromatography (HPLC)

- The purification takes place in a separation column between a stationary and a mobile phase.
- The stationary phase is a granular material with very small porous particles in a separation column.
- The mobile phase, on the other hand, is a solvent or solvent mixture which is forced at high pressure through the separation column.
- Via a valve with a connected sample loop, i.e. a small tube or a capillary made of stainless steel, the sample is injected into the mobile phase flow from the pump to the separation column using a syringe.

- Subsequently, the individual components of the sample migrate through the column at different rates because they are retained to a varying degree by interactions with the stationary phase.
- After leaving the column, the individual substances are detected by a suitable detector and passed on as a signal to the HPLC software on the computer.
- At the end of this operation/run, a chromatogram in the HPLC software on the computer is obtained.
- The chromatogram allows the identification and quantification of the different substances.

Instrumentation of High-Performance Liquid Chromatography (HPLC)



The Pump

- The development of HPLC led to the development of the pump system.
- The pump is positioned in the most upper stream of the liquid chromatography system and generates a flow of eluent from the solvent reservoir into the system.
- High-pressure generation is a "standard" requirement of pumps besides which, it should also to be able to provide a consistent pressure at any condition and a controllable and reproducible flow rate.
- Most pumps used in current LC systems generate the flow by back-and-forth motion of a motor-driven piston (reciprocating pumps). Because of this piston motion, it produces "pulses".

Injector

- An injector is placed next to the pump.
- The simplest method is to use a syringe, and the sample is introduced to the flow of eluent.
- The most widely used injection method is based on sampling loops.

- The use of the autosampler (auto-injector) system is also widely used that allows repeated injections in a set scheduled-timing.

Column

- The separation is performed inside the column.
- The recent columns are often prepared in a stainless steel housing, instead of glass columns.
- The packing material generally used is silica or polymer gels compared to calcium carbonate.
  The eluent used for LC varies from acidic to basic solvents.
- Most column housing is made of stainless steel since stainless is tolerant towards a large variety of solvents.

Detector

- Separation of analytes is performed inside the column, whereas a detector is used to observe the obtained separation.
- The composition of the eluent is consistent when no analyte is present. While the presence of analyte changes the composition of the eluent. What detector does is to measure these differences.
- This difference is monitored as a form of an electronic signal. There are different types of detectors available.

Recorder

- The change in eluent detected by a detector is in the form of an electronic signal, and thus it is still not visible to our eyes.
- In older days, the pen (paper)-chart recorder was popularly used. Nowadays, a computer-based data processor (integrator) is more common.
- There are various types of data processors; from a simple system consisting of the in-built printer and word processor while those with software that are specifically designed for an LC system which not only data acquisition but features like peak-fitting, baseline correction, automatic concentration calculation, molecular weight determination, etc.

Degasser

The eluent used for LC analysis may contain gases such as oxygen that are non-visible to our eyes.

- When gas is present in the eluent, this is detected as noise and causes an unstable baseline.
- Degasser uses special polymer membrane tubing to remove gases.
- The numerous very small pores on the surface of the polymer tube allow the air to go through while preventing any liquid to go through the pore.

Column Heater

The LC separation is often largely influenced by the column temperature.

- In order to obtain repeatable results, it is important to keep consistent temperature conditions.
- Also for some analysis, such as sugar and organic acid, better resolutions can be obtained at elevated temperatures (50 to 80°C).

- Thus columns are generally kept inside the column oven (column heater).

Types of High-Performance Liquid Chromatography (HPLC)

1. **Normal phase:**

Column packing is polar (e.g silica) and the mobile phase is non-polar. It is used for water-sensitive compounds, geometric isomers, cis-trans isomers, and chiral compounds.

2. **Reverse phase:**

The column packing is non-polar (e.g C18), the mobile phase is water+ miscible solvent (e.g methanol). It can be used for polar, non-polar, ionizable and ionic samples.

3. **Ion exchange:**

Column packing contains ionic groups and the mobile phase is buffer. It is used to separate anions and cations.

4. **Size exclusion:**

Molecules diffuse into pores of a porous medium and are separated according to their relative size to the pore size. Large molecules elute first and smaller molecules elute later.

Applications of High-Performance Liquid Chromatography (HPLC)

The HPLC has developed into a universally applicable method so that it finds its use in almost all areas of chemistry, biochemistry, and pharmacy.

- Analysis of drugs
- Analysis of synthetic polymers
- Analysis of pollutants in environmental analytics
- Determination of drugs in biological matrices
- Isolation of valuable products
- Product purity and quality control of industrial products and fine chemicals
- Separation and purification of biopolymers such as enzymes or nucleic acids
- Water purification
- Pre-concentration of trace components
- Ligand-exchange chromatography
- Ion-exchange chromatography of proteins
- High-pH anion-exchange chromatography of carbohydrates and oligosaccharides

Advantages of High-Performance Liquid Chromatography (HPLC)

1. Speed
2. Efficiency
3. Accuracy

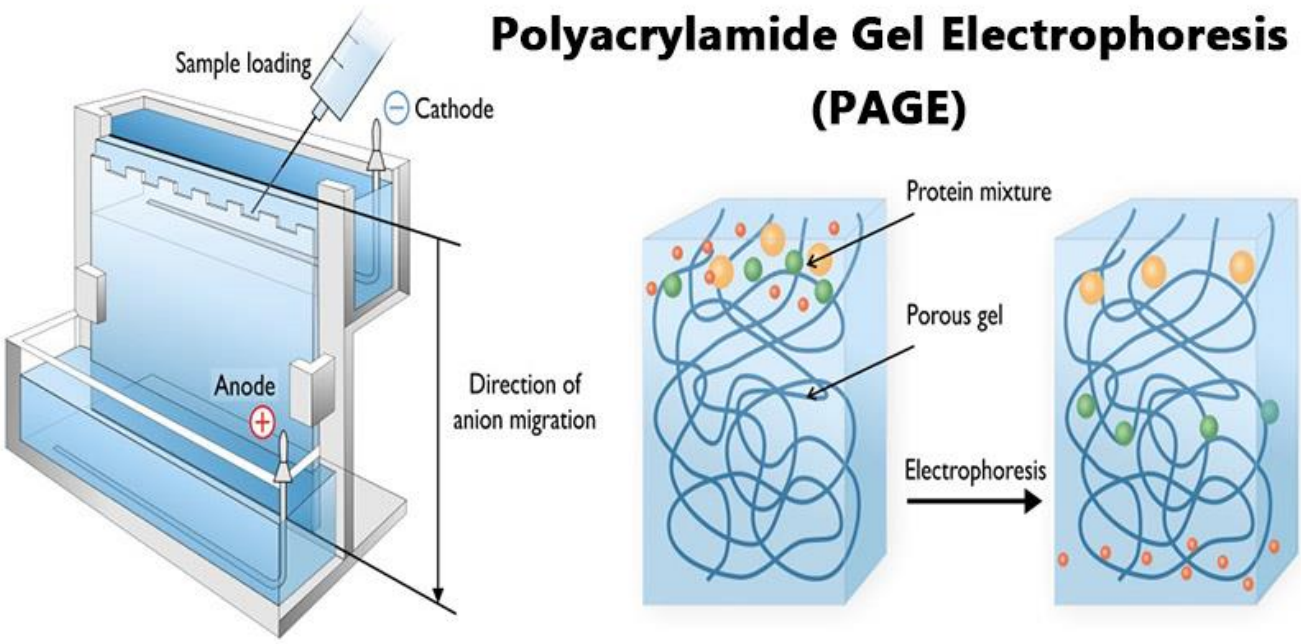
4. Versatile and extremely precise when it comes to identifying and quantifying chemical components.

Limitations

1. **Cost:** Despite its advantages, HPLC can be costly, requiring large quantities of expensive organics.
2. **Complexity**
3. HPLC does have **low sensitivity** for certain compounds, and some cannot be detected as they are irreversibly adsorbed.
4. Volatile substances are better separated by gas chromatography.

Polyacrylamide Gel Electrophoresis (PAGE)

- Electrophoresis through **agarose** or polyacrylamide gels is a standard method used to separate, identify and purify biopolymers, since both these gels are porous in nature.
- Polyacrylamide gels are chemically cross-linked gels formed by the polymerization of acrylamide with a cross-linking agent, usually N,N'-methylenebisacrylamide.
- The reaction is a free radical polymerization, usually carried out with ammonium persulfate as the initiator and N,N,N',N'-tetramethylethylendiamine (TEMED) as the catalyst.
- Polyacrylamide gel electrophoresis (PAGE) is a technique widely used in biochemistry, forensic chemistry, genetics, molecular biology and biotechnology to separate biological macromolecules, usually proteins or nucleic acids, according to their electrophoretic mobility.
- The most commonly used form of polyacrylamide gel electrophoresis is the Sodium dodecyl suplhate Polyacrylamide gel electrophoresis (SDS- PAGE) used mostly for the separation of proteins.



Principle of Polyacrylamide Gel Electrophoresis (PAGE)

SDS-PAGE (Polyacrylamide Gel Electrophoresis), is an analytical method used to separate components of a protein mixture based on their size.

The technique is based upon the principle that a charged molecule will migrate in an electric field towards an electrode with opposite sign. The general electrophoresis techniques cannot be used to determine the molecular weight of biological molecules because the mobility of a substance in the gel depends on both charge and size.

To overcome this, the biological samples needs to be treated so that they acquire uniform charge, then the electrophoretic mobility depends primarily on size. For this different protein molecules with different shapes and sizes, needs to be denatured (done with the aid of SDS) so that the proteins lose their secondary, tertiary or quaternary structure .The proteins being covered by SDS are negatively charged and when loaded onto a gel and placed in an electric field, it will migrate towards the anode (positively charged electrode) are separated by a molecular sieving effect based on size. After the visualization by a staining (protein-specific) technique, the size of a protein can be calculated by comparing its migration distance with that of a known molecular weight ladder (marker).

Requirements for Polyacrylamide Gel Electrophoresis (PAGE)

- Acrylamide solutions (for resolving & stacking gels).

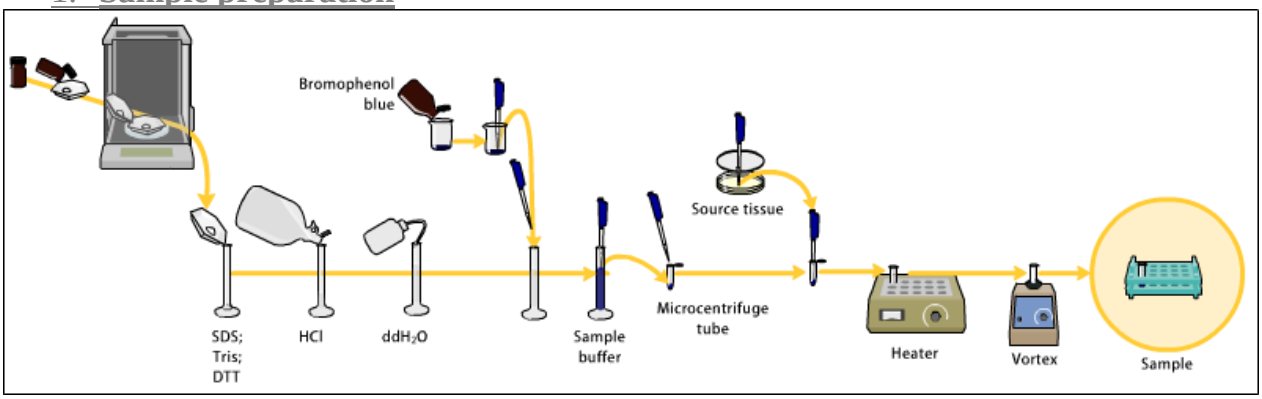
- Isopropanol / distilled water.
- Gel loading buffer.
- Running buffer.
- Staining, destaining solutions.
- Protein samples
- Molecular weight markers.

The equipment and supplies necessary for conducting SDS-PAGE includes:

- An electrophoresis chamber and power supply.
- Glass plates (a short and a top plate).
- Casting frame
- Casting stand
- Combs

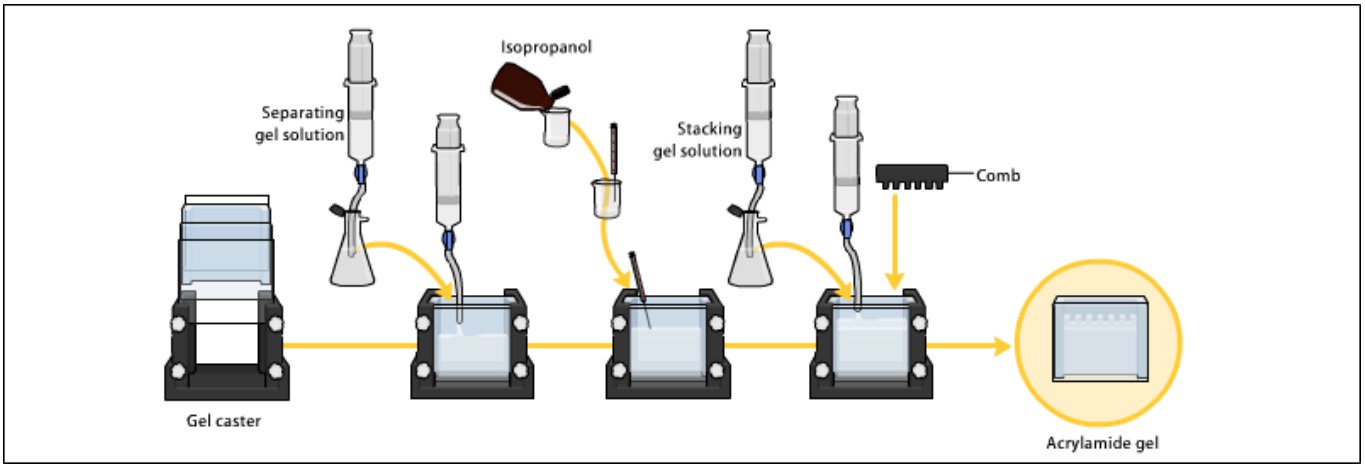
Steps Involved in Polyacrylamide Gel Electrophoresis (PAGE)

1. **Sample preparation**



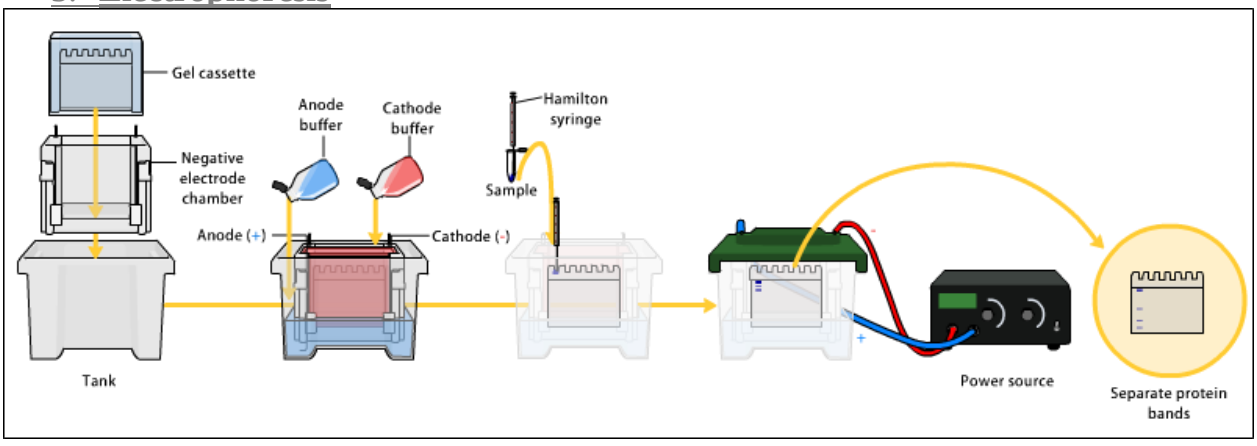
- Samples may be any material containing proteins or nucleic acids.
- The sample to analyze is optionally mixed with a chemical denaturant if so desired, usually SDS for proteins or urea for nucleic acids.
- SDS is an anionic detergent that denatures secondary and non–disulfide–linked tertiary structures, and additionally applies a negative charge to each protein in proportion to its mass. Urea breaks the hydrogen bonds between the base pairs of the nucleic acid, causing the constituent strands to anneal. Heating the samples to at least 60 °C further promotes denaturation.
- A tracking dye may be added to the solution. This typically has a higher electrophoretic mobility than the analytes to allow the experimenter to track the progress of the solution through the gel during the electrophoretic run.

2. **Preparation of polyacrylamide gel**



- The gels typically consist of acrylamide, bisacrylamide, the optional denaturant (SDS or urea), and a buffer with an adjusted pH.
- The ratio of bisacrylamide to acrylamide can be varied for special purposes, but is generally about 1 part in 35. The acrylamide concentration of the gel can also be varied, generally in the range from 5% to 25%.
- Lower percentage gels are better for resolving very high molecular weight molecules, while much higher percentages of acrylamide are needed to resolve smaller proteins,
- Gels are usually polymerized between two glass plates in a gel caster, with a comb inserted at the top to create the sample wells.
- After the gel is polymerized the comb can be removed and the gel is ready for electrophoresis.

3. **Electrophoresis**



- Various buffer systems are used in PAGE depending on the nature of the sample and the experimental objective.
- The buffers used at the anode and cathode may be the same or different.

- An electric field is applied across the gel, causing the negatively charged proteins or nucleic acids to migrate across the gel away from the negative and towards the positive electrode (the anode).
- Depending on their size, each biomolecule moves differently through the gel matrix: small molecules more easily fit through the pores in the gel, while larger ones have more difficulty.
- The gel is run usually for a few hours, though this depends on the voltage applied across the gel.
- After the set amount of time, the biomolecules will have migrated different distances based on their size.
- Smaller biomolecules travel farther down the gel, while larger ones remain closer to the point of origin.
- Biomolecules may therefore be separated roughly according to size, which depends mainly on molecular weight under denaturing conditions, but also depends on higher-order conformation under native conditions.

4. **Detection**

- Following electrophoresis, the gel may be stained (for proteins, most commonly with Coomassie Brilliant Blue or autoradiography; for nucleic acids, ethidium bromide; or for either, silver stain), allowing visualization of the separated proteins, or processed further (e.g. Western blot).
- After staining, different species biomolecules appear as distinct bands within the gel.
- It is common to run molecular weight size marker sof known molecular weight in a separate lane in the gel to calibrate the gel and determine the approximate molecular mass of unknown biomolecules by comparing the distance traveled relative to the marker.

Applications of Polyacrylamide Gel Electrophoresis (PAGE)

- Measuring molecular weight.
- Peptide mapping.
- Estimation of protein size.
- Determination of protein subunits or aggregation structures.
- Estimation of protein purity.
- Protein quantitation.
- Monitoring protein integrity.
- Comparison of the polypeptide composition of different samples.
- Analysis of the number and size of polypeptide subunits.
- Post-electrophoresis applications, such as Western blotting.
- Staining of Proteins in Gels with Coomassie G-250 without Organic Solvent and Acetic Acid.
- Pouring and Running a Protein Gel by reusing Commercial Cassettes.

- Selective Labelling of Cell-surface Proteins using CyDye DIGE Fluor Minimal Dyes.
- Detection of Protein Ubiquitination.

Advantages of Polyacrylamide Gel Electrophoresis (PAGE)

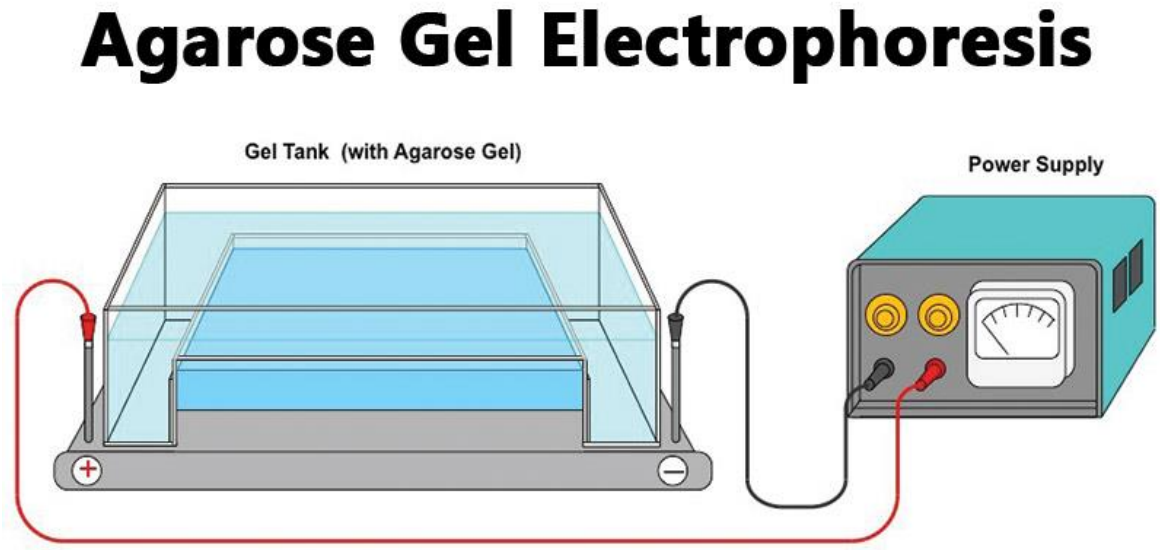
- Stable chemically cross-linked gel
- Greater resolving power (Sharp bands)
- Can accommodate larger quantities of DNA without significant loss in resolution
- The DNA recovered from polyacrylamide gels is extremely pure
- The pore size of the polyacrylamide gels can be altered in an easy and controllable fashion by changing the concentrations of the two monomers.
- Good for separation of low molecular weight fragments

Disadvantages of Polyacrylamide Gel Electrophoresis (PAGE)

- Generally more difficult to prepare and handle, involving a longer time for preparation than agarose gels.
- Toxic monomers
- Gels are tedious to prepare and often leak
- Need new gel for each experiment Stable chemically cross-linked gel

Agarose Gel Electrophoresis

- Agarose gel electrophoresis is a method of gel electrophoresis used in biochemistry, molecular biology, genetics, and clinical chemistry to separate a mixed population of macromolecules such as DNA , RNA or proteins in a matrix of agarose.
- Agarose is a natural linear polymer extracted from seaweed that forms a gel matrix by hydrogen-bonding when heated in a buffer and allowed to cool.
- They are the most popular medium for the separation of moderate and large-sized nucleic acids and have a wide range of separation.



Principle of Agarose Gel Electrophoresis

Gel electrophoresis separates DNA fragments by size in a solid support medium such as an agarose gel. Sample (DNA) are pipetted into the sample wells, followed by the application of an electric current at the anodal, negative end which causes the negatively-charged DNA to migrate (electrophorese) towards the bottom (cathodal, positive) end. The rate of migration is proportional to size: smaller fragments move more quickly, and wind up at the bottom of the gel.

DNA is visualized by including in the gel an intercalating dye, ethidium bromide. DNA fragments take up the dye as they migrate through the gel. Illumination with ultraviolet lightc auses the intercalated dye to fluoresce.

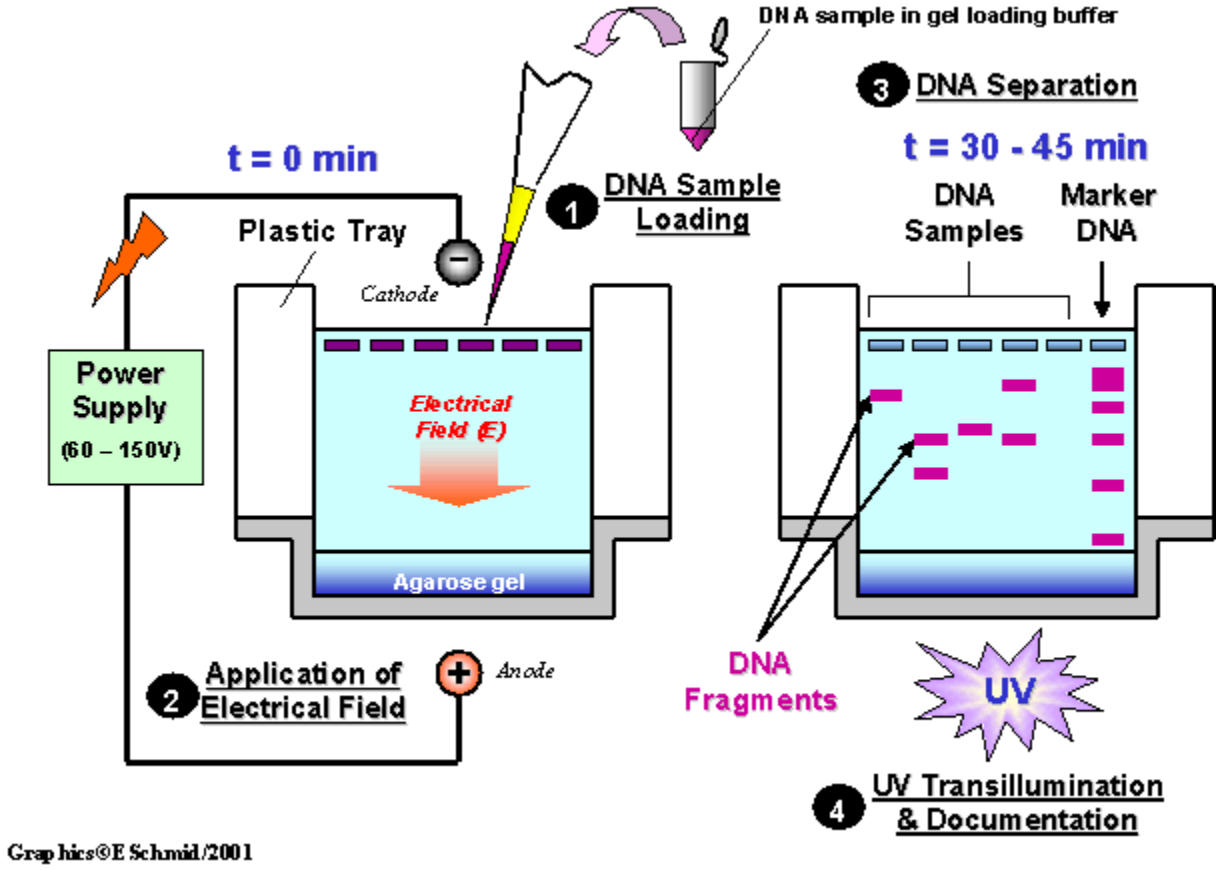
The larger fragments fluoresce more intensely. Although each of the fragments of a single class of molecule are present in equimolar proportions, the smaller fragments include less mass of DNA, take up less dye, and therefore fluoresce less intensely. A "ladder" set of DNA fragments of known size can be run simultaneously and used to estimate the sizes of the other unknown fragments.

Requirements/ Instrumentation of Agarose Gel Electrophoresis

The equipment and supplies necessary for conducting agarose gel electrophoresis are relatively simple and include:

1. An **electrophoresis chamber** and **power supply**
2. **Gel casting trays**, which are available in a variety of sizes and composed of UVtransparent plastic. The open ends of the trays are closed with tape while the gel is being cast, then removed prior to electrophoresis.
3. **Sample combs**, around which molten medium is poured to form sample wells in the gel.
4. **Electrophoresis buffer**, usually Tris-acetate-EDTA (TAE) or Tris-borate-EDTA (TBE).
5. **Loading buffer**, which contains something dense (e.g. glycerol) to allow the sample to "fall" into the sample wells, and one or two tracking dyes, which migrate in the gel and allow visual monitoring or how far the electrophoresis has proceeded.
6. **Staining**: DNA molecules are easily visualized under an ultraviolet lamp when electrphoresed in the presence of the extrinsic fluor ethidium bromide. Alternatively, nucleic acids can be stained after electrophoretic separation by soaking the gel in a solution of ethidium bromide. When intercalated into doublestranded DNA, fluorescence of this molecule increases greatly. It is also possible to detect DNA with the extrinsic fluor 1-anilino 8-naphthalene sulphonate.
7. **Transilluminator** (an ultraviolet light box), which is used to visualize stained DNA in gels.

Steps Involved in Agarose Gel Electrophoresis



1. To prepare gel, agarose powder is mixed with electrophoresis buffer to the desired concentration, and heated in a microwave oven to melt it.

The concentration of Agarose Gel

- The percentage of agarose used depends on the size of fragments to be resolved.
- The concentration of agarose is referred to as a percentage of agarose to volume of buffer (w/v), and agarose gels are normally in the range of 0.2% to 3%.
- The lower the concentration of agarose, the faster the DNA fragments migrate.
- In general, if the aim is to separate large DNA fragments, a low concentration of agarose should be used, and if the aim is to separate small DNA fragments, a high concentration of agarose is recommended.

2. Ethidium bromide is added to the gel (final concentration 0.5 ug/ml) to facilitate visualization of DNA after electrophoresis.
3. After cooling the solution to about 60oC, it is poured into a casting tray containing a sample comb and allowed to solidify at room temperature.
4. After the gel has solidified, the comb is removed, taking care not to rip the bottom of the wells.

5. The gel, still in plastic tray, is inserted horizontally into the electrophoresis chamber and is covered with buffer.
6. Samples containing DNA mixed with loading buffer are then pipetted into the sample wells, the lid and power leads are placed on the apparatus, and a current is applied.
7. The current flow can be confirmed by observing bubbles coming off the electrodes.
8. DNA will migrate towards the positive electrode, which is usually colored red, in view of its negative charge.
9. The distance DNA has migrated in the gel can be judged by visually monitoring migration of the tracking dyes like bromophenol blue and xylene cyanol dyes.

Applications of Agarose Gel Electrophoresis

Agarose gel electrophoresis is a routinely used method for separating proteins, DNA or RNA.

- Estimation of the size of DNA molecules
- Analysis of PCR products, e.g. in molecular genetic diagnosis or genetic fingerprinting
- Separation of restricted genomic DNA prior to Southern analysis, or of RNA prior to Northern analysis.
- The agarose gel electrophoresis is widely employed to estimate the size of DNA fragments after digesting with restriction enzymes, e.g. in restriction mapping of cloned DNA.
- Agarose gel electrophoresis is commonly used to resolve circular DNA with different supercoiling topology, and to resolve fragments that differ due to DNA synthesis.
- In addition to providing an excellent medium for fragment size analyses, agarose gels allow purification of DNA fragments. Since purification of DNA fragments size separated in an agarose gel is necessary for a number molecular techniques such as cloning, it is vital to be able to purify fragments of interest from the gel.

Advantages of Agarose Gel Electrophoresis

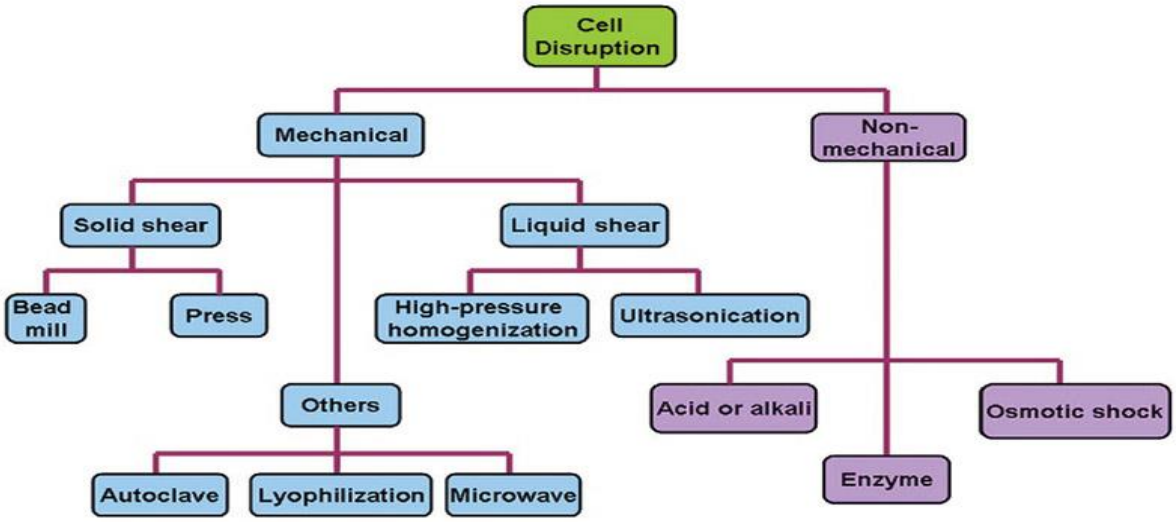
- For most applications, only a single-component agarose is needed and no polymerization catalysts are required. Therefore, agarose gels are simple and rapid to prepare.
- The gel is easily poured, does not denature the samples.
- The samples can also be recovered.

Disadvantages of Agarose Gel Electrophoresis

- Gels can melt during electrophoresis.
- The buffer can become exhausted.
- Different forms of genetic material may run in unpredictable forms.

Cell Disruption Methods

- Cell disruption is the process of obtaining intracellular fluid via methods that open the cell wall.
- The overall goal in cell disruption is to obtain the intracellular fluid without disrupting any of its components.
- The method used may vary depending on the type of cell and its cell wall composition.
- Irrespective of the method used, the main aim is that the disruption must be effective and the method should not be too harsh so that the product recovered remains in its active form.
- Cell disruption methods can be categorised into mechanical methods and non-mechanical methods.
- Mechanical methods are divided into solid shear methods and liquid shear methods.
- Non-mechanical methods can be divided into physical methods, chemical methods and enzymatic methods.



Mechanical Methods

Mechanical methods are those methods which required some sort of force to separate out intracellular protein without adding chemical or enzyme

1. Mortar & pastel/grinding
2. Blender
3. Bead beating
4. Ultra sonication
5. Homogenization

Mortar & Pestle

- It involves the grinding of the cells such that they are disrupted.

- This does not have to be in suspension and is often done with plant samples frozen in liquid nitrogen.
- When the material has been disrupted, metabolites can be extracted by adding solvents.

Blenders

- The use of blenders which employ high speed can be used to disrupt cell walls.
- It is the same process used by centrifugation, which separates or concentrates materials suspended in a liquid medium.

Bead beating

- Glass or ceramic beads are used to crack open cells
- The kind of mechanical shear is gentle enough to keep organelles intact.
- It can be used with all kinds of cells, just add beads to an equal amount of cell suspension and vortex.

Ultra sonication

- Ultrasonic homogenizers work by inducing vibration in a titanium probe that is immersed in the cell solution.
- A process called cavitation occurs, in which tiny bubbles are formed and explode, producing a local shockwave and disrupting cell walls by pressure change.
- This method is very popular for disruption of plant and fungal cells.

Homogenization

- Liquid-based homogenization is the most widely used cell disruption technique for small volumes and cultured cells.
- Cells are lysed by forcing the cell or tissue suspension through a narrow space
- Homogenizers use shearing forces on the cell similar to the bead method.
- Homogenization can be performed by squeezing cells through a tube that is slightly smaller than beads beating.

Non-Mechanical Methods

Non mechanical methods are further divided into three class which are following :

A. Physical methods

1. Freeze Thaw

- It is suitable when working with soft plant material and algae.
- Disruption is achieved via a series of freezing and thawing cycles.
- Freezing forms ice crystals, which expand upon thawing, and this ultimately causes the cell wall to rupture.

2. Microwave/ Thermolysis

- Microwave (along with autoclave and other high temperature methods) are used to disrupt the bonds within cell walls, and also to denature proteins.
- However, uncontrolled amount of heat can easily denature or damage target proteins and subtances.

3. Osmotic Shock

- Through the process of osmosis, water can be moved into the cell causing its volume to increase to the point that it bursts.
- The method however, can only work with animal cells and protozoa, since they do not have cell walls.

4. Electric Discharges

- It is also possible to achieve cell disruption via electrical discharges in mammalian and other cells that are bounded by plasma membranes only.

B. Chemical methods

- They are often used with plant cells (and sometimes in combination with shearing).
- Organic solvents such as toluene, ether, benzene, methanol, surfactants, and phenyl ethyl alcohol DMSO can be used to permeate cell walls.
- EDTA can be used specifically to disrupt the cell walls of gram negative bacteria, whose cell walls contain lipopolysaccharides that are stabilized by cations like Mg^{2+} and Ca^{2+}.
- EDTA will chelate the cations leaving holes in the cell walls.

3. **Enzymatic methods**

- Another strategy to achieve cell lysis is to use digestive enzymes which will decompose the microbial cell wall.
- Different cell types and strains have different kind of cell walls and membranes, and thus the used enzyme depends on microbe. For example, lysozyme is commonly used enzyme to digest cell wall of gram positive bacteria. Lysozyme hydrolyzes β-1-4-glucosidic bonds in the peptidoglycan.
- The cell wall of yeast and fungi differs significantly from the cell wall bacteria. One commonly used enzyme mixture for degradation of cell wall of yeast and fungi is Zymolyase.
- It has for example β-1,3 glucanase and β-1,3-glucan laminaripentao-hydrolase activities (Zymolyase | Yeast lytic enzyme).
- In addition, the enzymes that are commonly used for degradation of cell wall of yeast and fungi include different cellulases, pectinases, xylanases and chitinases.
- Enzymes such as beta(1-6) and beta(1-3) glycanases, proteases and mannase can also be used to disrupt the cell wall.

Significance

- Cell disruption is an essential part of biotechnology and the downstream processes related to the manufacturing of biological products.
- It is necessary for the extraction and retrieval of the desired products, as cell disruption significantly enhances the recovery of biological products.