BTY 401 INDUTRIAL BITECHNOLOGY

UNIT 1

Topic : recombinant proteins of prokaryotes and Eukaryotes

The advent of modern biotechnology has allowed for the optimization of production and extraction processes of bioactive molecules by expanding on the available organisms to be used as expression systems. Since the development of genetic engineering techniques that enabled the construction of recombinant organisms, production of heterologous proteins in cheaper, safer, and more manageable host cells has become a widespread practice in the industrial, food, and pharmaceutical fields. Because the specific protein of interest has specific characteristics that make certain systems more preferable, the proper selection of an organism for its optimal expression needs to take in account several parameters. Although the characterization of recombinant protein expression systems is far from complete, several of them have been far more studied and implemented, ranging from simple bacteria to complete multicellular organisms.

Prokaryotic Systems

Prokaryotes are, perhaps, the organisms most widely associated to the use of genetic engineering for production of specific biomolecules. They represent an ideal expression system in terms of their ease and low cost of culture maintenance, even in continuous operation, and their high growth rates. Because they can be grown in simple media, successful recombinant protein production in bacteria is usually a very cost-effective process.

The expression of foreign protein molecules in prokaryotic systems is a process with inherent differences from the protein's source organism. Naturally, these microorganisms are not able to carry out post-translational modifications on the protein product. These include molecule glycosylation, phosphorylation, acylation, methylation and addition of other chemical groups, as well as addition or cleavage of peptide units, chemical modification of amino acid residues, and formation of disulfide bridges. Since proteins from higher organisms might require such modifications for their adequate folding and performance of their function, their absence in a prokaryotic system might have a profound influence on their structure and activity, as well as their stability, resistance to degradation and even solubility on different media[1].

Another phenomenon to be considered is the need of certain proteins for chaperon molecules in order for their correct folding to be possible. The inability to achieve a correct structure, besides affecting function,

may result in a radical change in the molecule's solubility that results in the formation of inclusion bodies. Although their formation has been exploited, largely because it protects the product from proteolytic degradation and may enhance subsequent purification[2], inclusion bodies require solubilization steps[3], and the cell disruption required to extract the misfolded product also releases cytoplasmic components that become contaminants and constrain the purity levels that may be achieved[4]. Approaches to overcome their formation include the coexpression of chaperones[5], as well as using flexible promoters that avoid a high rate of expression that may induce their formation[6].

Problems might even be encountered on the amino acid sequence itself. Bacterial protein synthesis is initiated using the amino acid N-formylmethionine instead of the methionine found in higher organisms. Its presence can interfere with the protein's structure, besides eliciting an immune response if the product is to be used for medical purpose

Product purification and concentration can be, even so, greatly eased by expressing fusion proteins, where the target gene is fused to nucleotide sequences that are translated into short functional peptides, or 'tags'. Affinity tags, for example, facilitate downstream product recovery[12]. Fusion with reporter molecules allows for detection and characterization of the protein of interest[13]. Fusion products can also be used to confer the protein a greater resistance toproteases

An alternative prokaryotic expression system is *Bacillus subtilis*, which shares many of the desirable growth and production characteristics of *E. coli*, but displays some different properties. A noteworthy distinction is its ability to naturally become competent for transformation with foreign nucleic acids[19]. This soil-dwelling microorganism does not produce lipopolysaccharides, preventing the risk of toxins being present with the final product. Its genetics are also well- characterized, with many techniques developed for its manipulation, with the added value of being able to secrete proteins into the extracellular media.

Eukaryotic Systems

Yeasts provide an excellent expression system that shares the prokaryotes' potential for large-scale culture thanks to cheap and simple incubation conditions, as well as high growth rates, and yet displays biological characteristics unique to eukaryotes that enable the production of proteins requiring certain biochemical processes not offered by bacteria. Because they are, indeed, eukaryotes, yeasts hold a much greater potential to correctly fold and modify proteins in a manner similar to that undergone in the gene of interest's source organism. They are quite capable of secreting intracellular proteins into their culture medium, and they are also free of endotoxins, should their cells need to be ruptured. They pose an approach to eukaryotic gene expression with much more ease of culture and genetic engineering than

other eukaryotes.

Saccharomyces cerevisiae represents a fermentation system that leapt from the age-old bread and wine-making process onto recent vaccine production[29,30]. Other yeasts also used for recombinant protein expression include *Pichia pastoris*, *Schizosaccharomyces pombe*, *Arxula adeninivorans*, *Hansenula polymorpha*, *Kluyveromyces lactis*, and *Yarrowia lipolytica*[31,32,33], among others.

Although many of the processes involved in gene expression are shared between yeasts and higher eukaryotes, correct glycosylation remains a widespread problem when using them as expression systems. The glycosylation patterns exhibited by yeasts differ substantially from those of mammals, for example[34]. Approaches toward the humanization of glycosylation patterns in modified yeast strains have already been developed[35,36].

Transformation strategies in yeasts enable the integration of nucleotide sequences into their genome, which may result in increased genetic stability, insertion of longer DNA molecules, and expression of different peptides, as even transformation with multiple plasmids can be done[38]. Various strong promoters are available for gene expression in yeasts. Although less common than in prokaryotes, inclusion bodies can also be formed, especially in instances of high-level protein production, as in *P. pastoris*[38].

Like their unicellular counterparts, filamentous fungi can also be used for recombinant protein production. Although they are recognized as a system capable of high levels of protein secretion, the pathways involved are far less studied than in yeasts. Several members of the genera *Aspergillus, Trichoderma*, *Chrysosporium*, and *Fusarium* are recognized as viable expression systems[39], but general obstacles in expression in conventional culture have led to alternative techniques for protein production, like solid-state culture

The transformation strategy to be used in plant cells widely depends on the desired specificity of expression localization as well as characteristics and purpose of the target protein. Techniques used for this purpose include viral vectors and bioballistics, as well as the well-known *Agrobacterium tumefaciens*[53]. Inherent in the nature of the method of transformation are some disadvantages, like low predictability of insertion sites, difficulty in establishing genetically stable transgenic lineages, and relatively low transformation efficiency[54]. Other drawbacks include issues in safety for consumption and growth in the environment, as well as the ever-present challenge of different glycosylation patterns.

Alternative expression systems that aim to exploit the autotrophic capacity of certain organisms include the use of suspension cultures of plant cells[55], combining the advantages offered by the more

complex photosynthetic eukaryotes with the potential of large-scale suspension bioreactors, as well as the diverse range of microalgae species whose study has been on the rise in the last years for production of biomolecules[56].

Although time and frequency of usage has a profound relation with an expression system's characterization, the optimal organism for the production of a certain heterologous protein will vary depending on the target product's particular characteristics. New systems are constantly being developed and implemented as investigations on cellular processes are further accomplished, and innovation is certainly a factor to consider when selecting the proper expression system for a specific gene. Currently, there exists no universal expression system capable of assuming and executing each and every biological function that may be required for proper synthesis and modification of proteins. Feasibility, readiness and efficiency must all come together to adopt and adapt the proper organism for adequate expression quality and quantity.

Topic : Bioreactors and types

Bioreactors are vessels in which raw materials are converted to product by the action of living organisms. A bioreactor provides the optimal environment for cells and microorganisms to grow by maintaining pH, temperature, gas, and nutrient supply

Type # 1. Continuous Stirred Tank Bioreactors:

A continuous stirred tank bioreactor consists of a cylindrical vessel with motor driven central shaft that supports one or more agitators (impellers). The shaft is fitted at the bottom of the bioreactor (Fig. 19.1 A). The number of impellers is variable and depends on the size of the bioreactor i.e., height to diameter ratio, referred to as aspect ratio.

Advantages of STRs:

There are many advantages of STRs over other types. These include the efficient gas transfer to growing cells, good mixing of the contents and flexible operating conditions, besides the commercial availability of the bioreactors.

Type # 2. Bubble Column Bioreactors:

In the bubble column bioreactor, the air or gas is introduced at the base of the column through perforated pipes or plates, or metal micro porous spargers (Fig. 19.1B). The flow rate of the air/gas influences the performance factors —O_2 transfer, mixing. The bubble column bioreactors may be fitted with perforated plates to improve performance. The vessel used for bubble column bioreactors is usually cylindrical with an aspect ratio of 4-6 (i.e., height to diameter ratio).

Type # 3. Airlift Bioreactors:

In the airlift bioreactors, the medium of the vessel is divided into two interconnected zones by means of a baffle or draft tube. In one of the two zones referred to a riser, the air/gas is pumped. The other zone that receives no gas is the down comer. The dispersion flows up the riser zone while the down flow occurs in the down comer. Thereare two types of airlift bioreactors.

Internal-loop airlift bioreactor (Fig. 11.1C) has a single container with a central draft tube that creates interior liquid circulation channels. These bioreactors are simple in design, with volume and circulation at a fixed rate for fermentation.

External loop airlift bioreactor (Fig. 19.1D) possesses an external loop so that the liquid circulates through separate independent channels. These reactors can be suitably modified to suit the requirements of different fermentations. In general, the airlift bioreactors are more efficient than bubble columns, particularly for more denser suspensions of microorganisms. This is mainly because in these bioreactors, the mixingof the contents is better compared to bubble columns.

Airlift bioreactors are commonly employed for aerobic bioprocessing technology. They ensure a controlled liquid flow in a recycle system by pumping. Due to high efficiency, airlift bioreactors are sometimes preferred e.g., methanol production, waste water treatment, single-cell protein production. In general, the performance of the airlift bioreactors is dependent on the pumping (injection) of air and the liquid circulation.

Two-Stage airlift bioreactors:

Two-stage airlift bioreactors are used for the temperature dependent formation of products. Growing cells from one bioreactor (maintained at temperature 30°C) are pumped into another bioreactor (at temperature 42°C). There is a necessity for the two- stage airlift bioreactor, since it is very difficult to raise the temperature quickly from 30°C to 42°C in the same vessel. Each one of the bioreactors is fitted with valves and they are connected by a transfer tube and pump (Fig. 19.2A). The cells are grown in the first bioreactor and the bioprocess proper takes place in the second reactor.

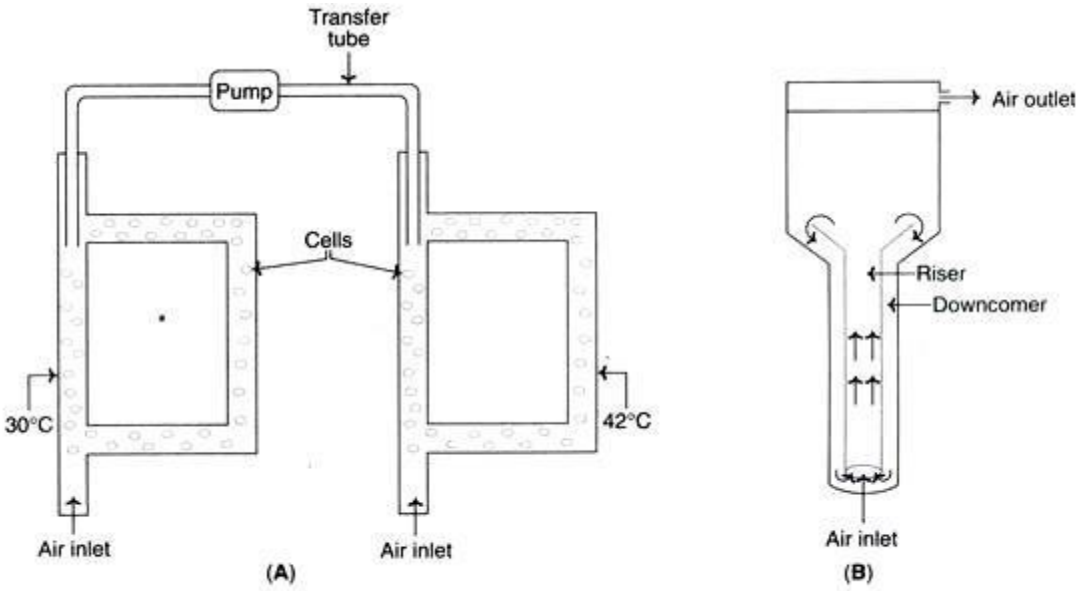


Fig. 19.2 : Types of bioreactors (A) Two–stage airlift bioreactor (B) Tower bioreactor.

Tower bioreactors:

A pressure-cycle fermenter with large dimensions constitutes a tower bioreactor (Fig. 19.2B). A high hydrostatic pressure generated at the bottom of the reactor increases the solubility of O_2 in the medium. At the top of the riser, (with expanded top) reduces pressure and facilitates expulsion of CO_2. The medium flows back in the down comer and completes the cycle. The advantage with tower bioreactor is that it has high aerationcapacities without having moving parts.

Type # 4. Fluidized Bed Bioreactors:

Fluidized bed bioreactor is comparable to bubble column bioreactor except the top position is expanded to reduce the velocity of the fluid. The design of the fluidized bioreactors (expanded top and narrow reaction column) is such that the solids are retained in the reactor while the liquid flows out (Fig. 19.3A). These bioreactors are suitable for use to carry out reactions involving fluid suspended biocatalysts such as immobilized enzymes, immobilized cells, and microbial flocs.

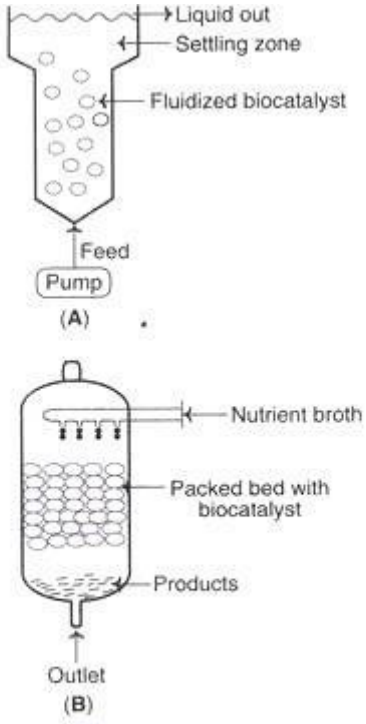


Fig. 19.3 : Types of bioreactors (A) Fluidized bed bioreactor (B) Packed bed bioreactor.

For an efficient operation of fluidized beds, gas is spared to create a suitable gas-liquid- solid fluid bed. It is also necessary to ensure that the suspended solid particles are not

too light or too dense (too light ones may float whereas to dense ones may settle at the bottom), and they are in a good suspended state. Recycling of the liquid is important to maintain continuous contact between the reaction contents and biocatalysts. This enable good efficiency of bioprocessing.

Type # 5. Packed Bed Bioreactors

A bed of solid particles, with biocatalysts on or within the matrix of solids, packed in a column constitutes a packed bed bioreactor (Fig. 19.3B). The solids used may be porous or non-porous gels, and they may be compressible or rigid in nature. A nutrient broth flows continuously over the immobilised biocatalyst. The products obtained in the packed bed bioreactor are released into the fluid and removed. While the flow of the fluid can be upward or downward, down flow under gravity is preferred.

The concentration of the nutrients (and therefore the products formed) can be increased by increasing the flow rate of the nutrient broth. Because of poor mixing, it is rather difficult to control the pH of packed bed bioreactors by the addition of acid or alkali.

However, these bioreactors are preferred for bioprocessing technology involving product-inhibited

reactions. The packed bed bioreactors do not allow accumulation of the products to any significant extent.

Type # 6. Photo-Bioreactors:

These are the bioreactors specialised for fermentation that can be carried out either by exposing to sunlight or artificial illumination. Since *artificial* illumination is expensive, only the outdoor photo-bioreactors are preferred. Certain important compounds are produced by employing photo-bioreactors e.g., p-carotene, asthaxanthin.

UNIT-2

Down stream processing?

The various stages of processing that occur after the completion of the fermentation or bioconversion stage, including separation, purification, and packaging of the product.

1. Filtration:

Filtrationisthemostcommonlyusedtechniqueforseparatingthebiomassandculturefiltrate.The efficiency of filtration depends on many factors— the size of the organism, presence of other organisms,viscosityofthemedium,andtemperature.Severalfilterssuchasdepthfilters,absolute filters, rotary drum vacuum filters and membrane filters are in use.

Depth Filters:

They are composed of a filamentous matrix such as glass wool, asbestos or filter paper. The particlesaretrappedwithinthematrixandthefluidpassesout.Filamentousfungicanberemoved by using depth filters.

AbsoluteFilters:

Thesefiltersarewithspecificporesizesthataresmallerthantheparticlestoberemoved.Bacteria from culture medium can be removed by absolute filters.

RotaryDrum VacuumFilters:

Thesefiltersarefrequentlyusedforseparationofbrothcontaining10- 40% solids (by volume) and particles in the size of 0.5-10µm. Rotary drumvacuumfiltershavebeensuccessfullyusedforfiltrationofyeast cells and

filamentous fungi. The equipment is simple with low power consumptionandiseasytooperate.Thefiltrationunitconsistsofa

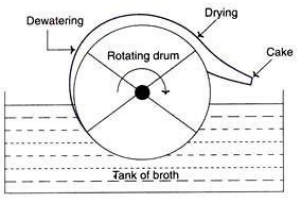
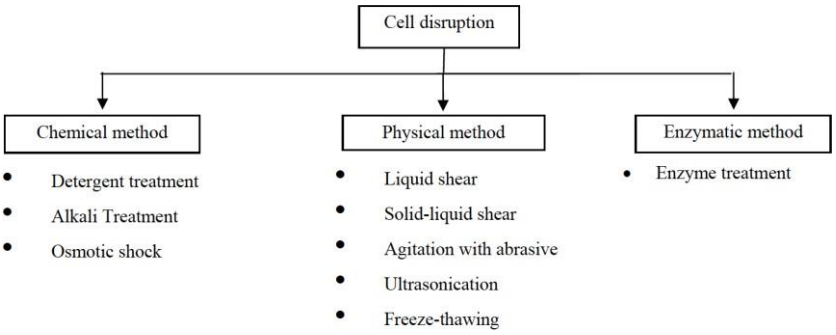


Fig. 20.2 : Diagrammatic representation of a rotary drum vacuum filter.

1. CellDisruption

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.Themethodusedmayvarydependingonthetypeofcellanditscellwall composition.Irrespectiveofthemethodused,themainaimisthatthedisruptionmustbeeffective, and the method should not be too harsh so that the product recovered remains in its active form.

Physicalmethodsofcelldisruption: Themicroorganismsorcellscanbedisruptedbycertain physicalmethods to release the intracellular products.



Ultra-sonication Ultrasonic disintegration is widelyemployed inthelaboratory. However, dueto highcost, itis not suitableforlarge-scaleusein industries.

Osmoticshock

Thismethodinvolvesthesuspensionofcells(freefromgrowthmedium)in20%bufferedsucrose. The cells are then transferred to water at about 4°C. Osmotic shock is used for the release of hydrolytic enzymes and binding proteins from Gram-negative bacteria.

Heatshock(thermolysis)

Breakage of cells by subjecting them to heat is relatively easy and cheap. But this technique can be used

only for a very few heat-stable intracellular products.

Highpressurehomogenization

This technique involves forcing of cell suspension at high pressure through a very narrow orifice to come out to atmospheric pressure. This sudden release of high pressure creates a liquid shear that can break the cells.

Impingement

Inthisprocedure,astreamofsuspendedcellsathighvelocityandpressureareforcedtohiteither astationarysurfaceorasecondstreamofsuspendedcells(impingeliterallymeanstostrikeorhit). The cells are disrupted by the forces created at the point of contact. Micro fluidizer is a device developed based on the principle of impingement. It has been successfully used for breaking E. coli cells. The advantage with impingement technique is that it can be effectively used for disrupting cells even at a low concentration.

Grindingwithglass beads

Thecellsmixedwithglassbeadsaresubjectedtoaveryhighspeedinareactionvessel.Thecells break as they are forced against the wall of the vessel by the beads. Several factors influence the cellbreakage-sizeandquantityoftheglassbeads,concentrationandageofcells,temperatureand agitatorspeed.Underoptimalconditions,onecanexpectamaximalbreakageofabout80%ofthe cells.

A diagrammatic representation of a cell disrupter employing glass beeds is shown in Fig. 20.6. It containsacylindricalbodywithaninlet,outletandacentralmotor-driven shaft.Tothisshaftare fittedradialagitators.Thecylinderisfittedwithglassbeads.Thecellsuspensionisaddedthrough theinletandthedisruptedcellscomeoutthroughtheoutlet.Thebodyofthecelldisrupteriskept cool while the operation is on.

Mechanicalandnon-mechanical methods

Among the physical methods of cell disruption described above, ultra-sonication, high-pressure homogenization,impingementandgrindingwithglassbeadsaremechanicalwhileosmoticshock and heat shock are non-mechanical. The chemical and enzymatic methods (described below) are non- mechanical in nature.

Chemicalmethods ofcelldisruption

Treatmentwithalkalies, organicsolventsanddetergentscanlysethe cellstoreleasethe contents.

Alkalies

Alkali treatment has been used for the extraction of some bacterial proteins. However, the alkali stability of the desired product is very crucial for the success of this method e.g., recombinant growth hormone can be efficiently released from E. coli by treatment with sodium hydroxide at pH 11.

Organicsolvents

Several water miscible organic solvents can be used to disrupt the cells e.g., methanol, ethanol, isopropanol,butanol.Thesecompoundsareinflammable;hencerequirespecialisedequipmentfor fire safety. The organic solvent toluene is frequently used. It is believed that toluene dissolves membrane phospholipids and creates membrane pores for release of intracellular contents.

Detergents

Detergentsthatareionicinnature,cationic-cetyltrimethylammoniumbromideoranionic-sodium lauryl sulfate can denature membrane proteins and lyse the cells. Non-ionic detergents (although less reactive than ionic ones) are also used to some extent e.g., Triton X-100 or Tween. The problem with the use of detergents is that they affect purification steps, particularly the salt precipitation. This limitation can beovercome by using ultrafiltration orion-exchangechromato- graphy for purification.

Enzymaticmethodsofcell disruption:

Celldisruptionbyenzymaticmethodshascertainadvantagesi.e.,lysisofcellsoccursundermild conditions in a selective manner. This is quite advantageous for product recovery. Lysozyme is themostfrequentlyusedenzymeandiscommerciallyavailable(producedfromheneggwhite).It

hydrolysesβ-1,4-glycosidicbondsofthemucopeptideinbacterialcellwalls.TheGram-positive bacteria (with high content of cell wall mucopeptides) are more susceptible for the action of lysozyme.

For Gram-negative bacteria, lysozyme in association with EDTA can break the cells. As the cell wallgetsdigestedbylysozyme,theosmoticeffectsbreaktheperiplasmicmembranetoreleasethe intracellular contents. Certain other enzymes are also used, although less frequently, for cell disruption. For the lysis of yeast cell walls, glucanase and mannanase in combination with proteases are used.

Combinationofmethods:

Inorder toincreasethe efficiencyofcelldisintegrationinacost-effective manner,a combination of physical, chemical and enzymatic methods is employed

2. Centrifugation:

The technique of centrifugation is based on the principle of density differences between the particlestobeseparated andthemedium.Thus,centrifugationismostlyusedforseparatingsolid particles from liquid phase (fluid/particle separation). Unlike the centrifugation that is conveniently carried out in the laboratory scale, there are certain limitations for large scale industrial centrifugation.

However, in recent years, continuous flow industrial centrifuges have been developed. There is a c sdepositedcanbe

r ermittently. The different es of centrifugesare picted in Fig. 20.4, anbriefly scribed hereunder

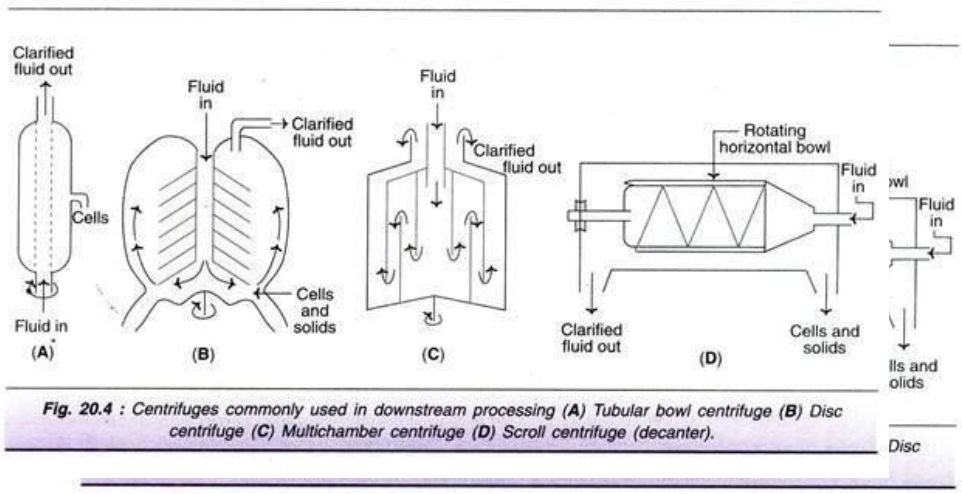


Fig. 20.4 : Centrifuges commonly used in downstream processing (A) Tubular bowl centrifuge (B) Disc centrifuge (C) Multichamber centrifuge (D) Scroll centrifuge (decanter).

Tubularbowlcentrifuge

This is a simple and a small centrifuge, commonly used in pilot plants. Tubular bowl centrifuge canbeoperatedatahighcentrifugalspeed,andcanberuninbothbatchorcontinuousmode.The solids are removed manually.

Disccentrifuge

Itconsistsofseveraldiscsthatseparatethebowlintosettlingzones.Thefeed/slurryisfedthrough a central tube. The clarified fluid moves upwards while the solids settle at the lower surface.

Multi-chambercentrifuge

Thisisbasicallyamodificationoftubularbowltypeofcentrifuge.Itconsistsofseveralchambers connected in such a way that the feed flows in a zigzag fashion. There is a variation in the centrifugal force in different

chambers. The force is much higher in the periphery chambers, as a result smallest particle settle down in the outermost chamber.

Scrollcentrifugeor decanter

It is composed of arotating horizontal bowl tapered at one end. The decanteris generally used to concentratefluidswithhighsolidconcentration(biomasscontent5-80%).Thesolidsaredeposited on the wall of the bowl which can be scrapped and removed from the narrow end.

3. Drying

Dryingisanessentialcomponentofproductformulation.Itbasicallyinvolvesthetransferofheat to a wet product for removal of moisture. Most of the biological products of fermentation are sensitive to heat, and therefore require gentle drying methods. Based on the method of heat transfer, drying devices may be categorized as contact, convection, radiation dryers. These three types of dryers are commercially available.

Spraydrying:

Spray dryingis used for dryinglargevolumes ofliquids. In spray drying,small droplets ofliquid containingtheproductarepassedthroughanozzledirectingitoverastreamofhotgas.Thewater evaporates and the solid particles are left behind.

Freeze-drying:

Freeze-dryingorlyophilizationisthemostpreferredmethodfordryingandformulationofawide- range of products—pharmaceuticals, foodstuffs, diagnostics, bacteria, viruses. This is mainly because freeze-drying usually does not cause loss of biological activity of the desired product.

Lyophilizationisbasedontheprincipleofsublimationofaliquidfromafrozenstate.Intheactual technique, the liquid containing the product is frozen and then dried in a freeze-dryer under vacuum. The vacuum can now be released and the product containing vials can be sealed e.g., penicillin can be freeze dried directly in ampules

Unit-III

Topic :production of Citric Acid

Citric acid, having the chemical formula C6H8O7, is a weak organic acid. Aspergillus species produce a huge range of metabolites, including citric acid.

- Citric acid is a naturally occurring acid. It is present in a number of fruits and vegetables. The citrus fruit with the highest concentration of citric acid is the lemon.
- Carl Wilhelm Scheele initially extracted citric acid from lemon juice in 1822 and defined its chemical composition.
- Citric acid is utilised in the food and beverage industries for numerous consumer goods. In soft drinks and syrups, as an acidulant, it stimulates a natural fruit flavour and imparts the necessary tartness.
- Citric acid is an intermediate organic component in the tricarboxylic acid (TCA) cycle and is naturally present in citrus fruits, pineapples, pears, and calcium citrate crystals. It is created mostly through fermentation.
- Citric acid generates several metallic salts, such as complexes with copper, iron, manganese, magnesium, and calcium.
- It is utilised as a sequestering agent in industrial processes and as an anticoagulant blood preservative due to the presence of these salts. In fats and oils, it decreases metal-catalyzed oxidation by chelating trace amounts of metals such as iron.

- Its function as a flavouring includes two components: first, its acidity, which leaves little aftertaste, and second, its capacity to accentuate other flavours.
- Citric acid is used as a scrubber to remove sulphur dioxide from flue gases, making a complex ion that then combines with H2S to produce elemental sulphur and regenerate citrate.
- This may become more significant as environmental forces increase.
- The triethyl, butyl, and acetyl tributyl esters of citric acid are employed as plasticizers in plastic films, and monostearyl citrate is utilised instead of citric acid as an antioxidant in oilsand fats.

Properties of Citric Acid – C6H8O7

C6H8O7	Citric Acid
Molecular Weight/ Molar Mass	192.124 g/mol
Density	1.66 g/cm³
Boiling Point	310 °C

Melting Point	153 °C

Numerous microorganisms are utilised in the production of citric acid. There are bacteria, fungi, and yeasts among these. However, A.Niger and saccharomycopsis sp. are utilised for commercial production due to its numerous benefits.

Microorganisms Used For Citric Acid Production

Bacteria

- Bacillus licheniformis
- Arthrobacter paraffinens
- Corynebacterium species

Yeasts

- Candida tropicalis

- C.oleophila
- C.guilliermondii
- C.Citroformans
- Hansenula anamola
- Yarrowia lipolytica

Fungi

- Aspergillus nagger
  A.aculeatus
- A.awamori

Production of Citric Acid / Fermentation of Citric Acid

The industrial production of citric acid can be conducted in three distinct ways:

1. Surface fermentation
2. Submerged fermentation
3. Solid-state fermentation

Production of Citric Acid / Fermentation of Citric Acid

1. Surface Culture Process for Production of Citric Acid

- This process is also known as "liquid surface fermentation." In 1919, surface culture fermentation was the first technique introduced for the synthesis of citric acid.

  In liquid surface fermentation, the culture medium (5-6 pH) is put to shallow, 5-20 cm-deep aluminium trays

- In the fermentation chamber, which ensures uniform air circulation and maintains relative temperature and humidity, the process is carried out.
- First, A.niger spores are blown onto the surface of the culture medium for approximately 5 to 6 days, followed by the passage of dry air.

- After 24 hours, the spores begin to germinate and white mycelium begins to form on the surface of the culture media.
- After the moulds have utilised the sugar content, the residual liquid is removed from the mycelial mat.

process of Surface Culture Method

This process consists of four phases:

1. Inoculum production,
2. Preparation of medium,
3. Fermentation process and
4. Harvest and recovery.

1. Inoculum Production

- Suspension of spores is employed as an inoculum in the synthesis of citric acid. From a stock culture, a suitable and high-yielding strain of A. niger is selected.
- Glass vials containing sporulating media are infected with the stock culture. At 25°C, the bottles are incubated for 10-14 days.
- Trace elements, such as manganese, zinc, and iron salts, must be appropriately maintained in the sporulating medium; otherwise, they will impact the output of citric acid during actual fermentation.
- Suspension of spores is achieved by suspending mature spores in an appropriate diluent, such as water containing the wetting ingredient sodium lauryl sulphate. In addition to the quantity, the viability of the spore crop is crucial

2. Preparation of Medium

- The medium used to produce citric acid must have a source of carbohydrates and inorganicsalts.
- As a carbon source, numerous substances can be utilised. However, sucrose and beet molasses are typically employed as carbon sources.
- Sucrose is the best carbon source among the evaluated organic compounds. A medium containing less than 15% sucrose is reported to have a high citric acid production.
- When sucrose is partially replaced by fructose or glucose, citric acid output is diminished.
- Commercially, beet molasses is widely employed as a carbon source in the manufacture of citric

acid by A. niger.

- In addition to sugars, beet molasses contains an abundance of inorganic salts. Before it is utilised in the creation of the medium, it is treated with ferrocyanide or ferricyanide to eliminate these excessive inorganic salts.
- The inorganic salts can also be extracted by passing the beet molasses through a cation exchange resin.
- In addition to carbon, elements such as nitrogen, potassium, phosphorus, and magnesium are required in the media.

  In minimal amounts, ammonium nitrate, potassium dihydrogen phosphate or potassium monohydrogen phosphate, and magnesium sulphate are added to the medium
- Higher concentrations of these components decrease the yield of citric acid and increase the output of oxalic acid.
- Adjusting the pH of the medium to 3.4-3.5 with hydrochloric acid is required. According to reports, a medium with a low pH facilitates less contamination, the synthesis of more citric acid, the suppression of the formation of oxalic acid, and simple sterilisation.
- In one litre of deionized water, salts and carbohydrates are dissolved. The medium must be sterilised at 55-103 to 69-103 Nm-2 per square inch of steam pressure for 30 minutes.

3. Fermentation Process

- The production media is placed in shallow pans in such a way that a 1 to 2.5 cm thick layer of medium is generated.
- The inoculum spores are introduced to the medium to maintain their floating state. This is accomplished through modulating devices.
- Incubation occurs in 30-40°C incubation chambers. Figure 4.3 depicts the standard fermentation setup.
- During fermentation, the temperature is held constant at 30 degrees Celsius. Air current ventilation is also essential for gas exchange, as the rate of citric acid generation decreases when CO_2 levels in the environment reach 10%.
- The germinating spores develop a thin layer of mycelium on the surface of the nutrient solution 24 hours after inoculation. Due to the incorporation of ammonium ions, the pH of the culture medium falls to between 1.5 and 2.0.
- If the iron concentration is high after 30 hours of fermentation, oxalic acid and yellow pigment are produced, which inhibits the recovery process.
- The mature mycelium floats as a thick, whitish, convoluted layer atop the liquid media. After

8-14 days, the fermentation ceases.

- The rate of sugar bioconversion to citric acid is proportional to the ratio of surface area to medium volume.
- If the ratio is reduced, the yield of citric acid will increase. In this shallow pan approach, the ratio of surface area to medium volume is reduced, which exposes a vast surface area of mycelial mat to a thin layer of medium.
- Under these conditions, sugar is increasingly transformed into citric acid. This is the reason why this method is considered superior to the submerged culture method. This method yields between 1.2 and 1.5 kilogramme of citric acid monohydrate per square metre of fermentation surface per hour.

Fermentation Process

4. Harvest and Recovery

- Separating the mycelium from the fermentation broth. By pressing the mycelium, any intracellular citric acid present in the mycelium can be extracted.
- Calcium hydroxide is used to treat the filtered broth. There is filtration and washing. The substance is then treated with an equivalent volume of sulphuric acid to produce citric acid. In this procedure, a precipitate of calcium sulphate is generated.
- Filtration is used to isolate the precipitate. By decolorizing and demineralizing an impure citric acid solution with activated carbon, an impure citric acid solution is created.
- Through evaporation, pure citric acid crystals are created. It is also extracted using a counter-current extraction technique.

2. Submerged Culture Process for Production of Citric Acid

- This process is also known as "Submerged culture fermentation." Approximately 80% of citric acid generation is accomplished through submerged fermentation.
- Submerged fermentation utilises black Aspergillus, also known as A. japonicus. It is conducted in a stainless steel bioreactor equipped with aeration, cooling system, impellers,etc.
- Substrates such as beet molasses, maize starch, etc. are used as carbon sources. As a source of nitrogen, ammonia is utilised.
- This approach requires pretreatment of the substrate, such as nutrition addition, sanitation,etc.
- The culture medium is injected with A. japonicus and maintained at 30 degrees Celsius.
- Typically, submerged fermentation is conducted in a batch bioreactor that can produce 1500 kg of citric acid and 500 kg of biomass from 2500 kg of glucose and 860 kg of oxygen.

- Important for production in the submerged culture process are three elements. They are the qualities of the metal used to make fermenters, mycelium structures, and oxygen delivery systems.
- Candida lipolytica, an alkane-utilizing fungus, can also be used in the continuous fermentation generation of citric acid. It yields 45% more citric acid than standard production.

Process of Submerged culture fermentation

1. Inoculum Production

- In this method, mycelial mats known as pellets are employed as inoculum for fermentation. From a stock culture, suitable and high-yielding strains of A. niger are selected.

A seed fermenter induces germination of the spores. In this seed fermenter, a nutrition solution containing 15% sugar from molasses is employed

- Cyanide ions are introduced to the medium to stimulate the production of mycelial pellets.
- The production of pellets is significantly influenced by the concentration of cyanide ions in the media. If cyanide ions are in lower concentration, citric acid production is diminished.
- Lower concentrations of cyanide ions encourage the production of regular mycelium rather than pellets.
- The spores germinate at 32 degrees Celsius and form 0.2 to 0.5 mm pellets within 24 hours. Throughout this time, the pH lowers to 4.3. These pellets are then employed as starter cultures in production fermenters.

2. Preparation of Medium

- The same medium used for surface culture is also used for this method.

3. Fermentation Process

- The majority of fermenters used for citric acid manufacturing have a capacity between 10 and 220 klt. They must be made from stainless steel to prevent heavy metal leaching.
- Normal steel, if used in the building of fermenters, may limit the synthesis of citric acid at pH levels between 1-2.
- Due to their huge surface-to-volume ratio, small stainless steel fermenters with a capacity of up to 1000 litres should be lined with plastic. Nevertheless, huge stainless steel fermenters do not require such a plastic lining.
- The mycelial pellets grown in the seed tank are transferred aseptically to the fermenters and

incubated at a constant temperature of 30 degrees Celsius.

- The structure of the mycelium that forms in the fermenter is essential to the success of the manufacturing process. If the mycelium is loose and filamentous with few branches and no chlamydospores, little citric acid is generated.
- The optimal amount of citric acid is produced when the mycelium is in pellet form. The iron-to-copper ratio in the media determines the type of the mycelium. In certain instances, production fermenters are directly inoculated with spores.
- Although A. niger has a low oxygen requirement, it is vulnerable to oxygen shortage. The oxygen concentration must be between 20 and 25 percent of the saturation value during the fermentation process.

3. Solid State Fermentation

- It is known as the "koji procedure." Japan is where the koji method was originally introduced. It relates to the utilisation of agro-industrial leftovers in the synthesis of citricacid.
- In the Koji process, common raw ingredients include apple pomace, sugar cane, and beet molasses, among others. Aspergillus niger makes use of raw resources.
- The pH and moisture content of the raw material are adjusted to 4-5 and 70%, respectively. Then, chill the raw material to temperatures between 30 and 60 degrees Celsius.
- Then, inoculate with A. niger. After inoculation, the medium is moved to big trays with a depth of 3-5 cm and incubated for 3-7 days at 25-30 degrees Celsius.
- The citric acid is finally collected from the fermenting vessel. The starch content of the raw material is converted to citric acid by the amylase enzyme of Aspergillus niger.
- Because trace elements have little effect on citric acid synthesis, the koji method does not require a pretreatment of the substrate.
- The solid substrate is saturated with water to a water content of 65-70 percent. Following the removal of superfluous water, the mass is subjected to a steaming procedure.

The surface of sterile starch paste is infected with Aspergillus niger conidia in the form of

- an aerosol or liquid conidia solution.
- The pH of the substrate is approximately 5-5.5, and the incubation temperature is 28-30 degrees Celsius.
- By adding Alpha-amylase, growth can be enhanced. Although the fungus can hydrolyze starch with its own alpha-amylase, it cannot do so efficiently. During citric acid synthesis, pH values went below 2

- Five to eight days are required for the solid-state surface procedure, after which the complete is removed with hot water. In some instances, citric acid is extracted from the cells using mechanical passes

Applications of Citric Acid

1. Food Additive

- Citric acid is used as a flavouring ingredient and preservative in food.
- It is utilised in processed foods such as beverages and soft drinks.
- Due to its sour flavour, it is utilised in the production of sour candies.
- Sometimes the sour candy is topped with a citric acid-based white powder.
- Several ice cream manufacturers utilise it as an emulsifier to keep fat globules at bay.

2. Cleaning Agent

- The acid citric is among the chelating agents.
- With the use of citric acid, limescale is eliminated from evaporators and boilers.
- The acid is utilised in soaps and laundry detergents because it softens water.
- In addition to citric acid, kitchen and bathroom cleansers also include citric acid.
- In addition to being a cleaning, it is also utilised as a deodorizer.

UNIT – 4

Topic: Enzyme purification and techniques

Enzymes area unit generally isolated from microorganism, animal, or plant sources. Microbial sources are particularly useful because they can be quickly grown into large colonies and are easy to store in vats prior to isolation. The separation and purification of enzymes require multiple separation steps involving filtration, centrifugation, chromatography, and more frequently bio-magnetic separation. The ability to use bio-magnetic separation for large volumes is a relatively new development in the field. Modern bio-magnetic separation racks that provide homogeneous magnetic force throughout the working volume are a drastic improvement over traditional setups and have made it possible to scale up the technique for use with large production volumes. This in-batch consistency is also important for process validation to ensure that products are reliable and safe for consumption.

Some Examples Of Industrial Uses Of Enzymes:

- Rennin for coagulation of milk to make cheese.
- Invertase from yeast and lactase in the food industry.
- Cellulase and amylase to remove waxes, oils, and starch coatings on fabrics and to improve the look of the final product.
- Amylase and protease for baking.
- Lipases in fruit juices to break down cell walls for increased yield.
- Proteases, lipases, amylases, oxidases, peroxidases, and cellulases in detergents to help break down stains and chemical bonds.
- Carbohydrase to convert starch into corn syrup.
- Zymase to convert carbohydrates into ethanol in alcoholic beverages.
- Cellulases are used to convert cellulose into glucose to improve biofuel yield.
- Lipase and phospholipase are used in the production of biodiesel by converting free fatty acids to fatty acid methyl esters.
- Phytases to improve agricultural feed efficiency.

Applications Of Enzymes

The biocatalysts (enzymes and cells) are used in multifarious ways in a different field. They grouped the applications into four broad categories: (i) therapeutic uses, (ii) analytical uses, (iii) manipulative uses, and (iv) industrial uses of enzymes.

Therapeutic Uses Of Enzymes

Enzymes are used for this purpose where some inborn errors of metabolism occur due to missing of an enzyme where specific genes are introduced to encode specific missing enzymes. However, in most cases, certain diseases are treated by administering the appropriate enzyme. For example, virilisation of disease developed due to loss of hydroxylase enzyme from adrenal cortex and introduction of the hydroxyl group (-OH) on 21-carbon of the ring structure of steroid hormone.

Steroids are compounds having a common skeleton in the form of perhydro-1, 2-cyclo-pentano-phenanthrene. The missing enzyme synthesises aldosterone (male hormone) in excess leading to masculinization of female baby and precocious sexual activity in males in about 5-7 years.

Similarly, treatment of leukaemia (a disease in which leukemic cells require exogenous asparagine for their growth) could be done by administering asparaginase of bacterial origin.

Analytical Uses Of Enzymes

Use of enzymes for analytical purposes is also important. Generally, endpoint and kinetic analysis are possible. Endpoint analysis refers to the total conversion of substrates into products in the presence of enzymes in a few minutes while kinetic analysis involves the rate of reaction and substrate/product concentration. Moreover, the analysis of antibodies, immuneglobins, necessary for human use poses a great promise. The usable enzymes are alkaline phosphatase, b-galactosidase, b-lactamase, etc. Another use of enzyme is in biosensor, device of biologically active material displaying characteristic specificity with chemical or electronic sensor to convert a biological compound into an electronic signal. It is constructed to measure almost anything from blood glucose. A simple carbon electrode, an ion-sensitive electrode, oxygen electrode or a photocell, maybe a sensor.

Manipulative Uses Of Enzymes

A variety of enzymes isolated from different sources are nowadays applied in genetic engineering as one of the biological tools.

Industrial Uses Of Enzymes

Enzymes are used in industries in different ways.

In Dairy industry

For a long time, the dairy enzyme calf rennet has been used in the dairy industry. In recent years, calf rennets are replaced by microbial rennets (e.g. Mucor michei). They are acid aspartate proteases. They slightly differ from calf rennets as they depend for reaction with casein on Ca++, temperature, pH, etc.

Lactase (produced by Bacillus stearothermophilus) is used for hydrolysis of lactose in whey or milk, and lipase for flavour development in special cheeses.

In Detergent Industry

During normal washing proteinaceous dirt often precipitates on solid cloths and proteins facilitate to adhere the dirt on textile fibres and make stains on cloths. These stains are difficult to remove from clothes. Nevertheless, it can be easily removed by adding proteolytic enzymes to the detergent. It attacks peptide bonds and therefore, dissolves protein. The alkaline serine protease obtained from B. licheniformis is most widely preferred to use in detergent. In addition, the serine protease of B. amyloliquefaciens is also used for this purpose. It contains a-amylase, hence to some extent it may be advantageous.

In Starch Industry

It has been mentioned earlier that hydrolysis of starch began in the early 1960s to prepare dextrose and glucose syrups. Furthermore, for complete acid hydrolysis of starch to dextrose glucoamylase was coupled with bacterial a-amylase. Currently, various enzymatic processes are applied to various products.

Glucose isomerase is an important enzyme used commercially in the conversion of glucose to fructose via isomerisation. Fructose is used in the preparation of fructose syrup.

The reaction mixture at the end contains 42% fructose, 52% glucose, and 6% dextrins. The mixture is sweeter than glucose and as sweet as sucrose. Now, techniques have been developed to obtain 55% fructose concentration in syrup.

In Brewing Industry

Enzymes used in the brewing industry are a-amylase, b-glucanase and protease which are required for malt in substitution of barley. Source of these enzymes is B. amyloliquefaciens. a-amylase is not required for liquefaction or brewing adjuncts and b-glucanase alleviates filtration problems due to poor malt quality and neutral protease helps in the inhibition of alkaline protease by an inhibitor.

In Wine Industry

Pectic enzymes are utilised in the wine business for top yield of product of improved quality. The pectic enzymes are pectin transeliminase (PTE), polymethyl galacturonase (PMG), polygalacturonases (PG), pectin esterase (PE), etc. However, cellulose enzymes provide a smart result once combined with different enzymes e.g. protease glucoamylase, etc.

Description

Enzymes are biological catalysts that play a crucial role in various metabolic processes in living organisms. Substrates are the molecules that enzymes may interact upon, and the enzyme changes the substrates into other molecules known as products. They are widely used in industries such as food, pharmaceutical, and biotechnology. However, enzymes obtained from natural sources are often impure and require purification to be used effectively in industrial applications. Enzyme purification is a process of separating and isolating enzymes from other cellular components to obtain pure enzymes. Producing the maximum yield of

the required enzyme with the highest catalytic activity and highest purity is the objective when choosing a purification technique.

Techniques for enzyme purification

Precipitation: Precipitation is the simplest and most commonly used technique for enzyme purification. It involves adding a salt or organic solvent to the enzyme solution, which causes the protein to precipitate out of solution. Then, centrifugation or filtering is used to collect the precipitate. This technique is suitable for enzymes that are relatively stable under the conditions required for precipitation.

Chromatography: Chromatography is a powerful technique for separating and purifying proteins. It involves passing a mixture of proteins through a column containing a solid matrix. The matrix is designed to interact selectively with different proteins, allowing them to be separated based on their physicochemical properties such as size, charge, and hydrophobicity. Different types of chromatography, such as ion exchange, size exclusion, and affinity chromatography, can be used depending on the properties of the enzyme to be purified.

Electrophoresis: Electrophoresis is a technique that separates proteins based on their charge and size using an electric field. It involves placing the enzyme solution on a gel matrix and applying an electric field. Proteins migrate through the gel based on their charge and size, allowing them to be separated and purified. This technique is commonly used in conjunction with other purification techniques such as chromatography.

Importance of enzyme purification

Enzyme purification is essential for various industrial applications of enzymes. The impurities present in the enzyme solution can affect the enzyme's stability, activity, and specificity, which can, in turn, affect the final product's quality and yield. Purified enzymes are more stable and active, and their specificity is higher, making them more efficient and effective in industrial processes.

Enzyme purification also allows for the study of enzyme's structure and function, which can aid in the development of new enzymes with improved properties. Understanding the structure and function of enzymes is critical for developing enzyme-based technologies that can contribute to sustainable industrial processes.

Topic: Biosensors:

- Analytical devices that consists a combination of biological detecting elements like sensor system and a transducer is termed as **biosensor.**

- Biosensors can be defined as self-sufficient integrated devices that has capacity to provide specific qualitative or semi-quantitative analytical information using a biological recognition element which is in direct-spatial contact with a transductional element.
- In simple words, biosensors are analytical devices that detects changes in biological processes and transform the biological data into electrical signal.
- The main features of biosensors are:
    - **Stability**
    - **Economical**
    - **Sensitivity**
    - **Reproducibility**

Components of biosensor:

- The block diagram of the biosensor consists of three segments namely, **sensor**, **transducer**, and **electrical circuit.**
    - **i. Sensor** or **detector:** The first segment is the sensor or detector which is a biological component. it is a biochemical receptor. It interacts with the analyte and signal the change in its composition as electrical signal.
    - **ii. Transducer:** The second segment is the transducer and it is a physical component which amplifies the biochemical signal received from detector, alters the resulting signal into electrical and displays in an attainable way.
    - **iii. Electrical circuit:** It is the associated part which consists of Signal Conditioning Unit, a Processor or Micro-controller and a Display Unit.

Principle of Biosensors:

- Biosensors works on the principle of signal transduction and biorecognition of element.
- All the biological materials including-enzyme, antibody, nucleic acid, hormone, organelle or whole cell can be used as sensor or detector in a device. But the desired bio-receptor is usually a specific deactivated enzyme.
- The deactivated enzyme is placed in proximity to the transducer.
- The tested analyte links to the specific enzyme (bio-receptor) and inducing a change in biochemical property of enzyme. The change in in turn gives an electronic response through an electroenzymatic approach.
- Electroenzymatic process is the chemical process of converting the enzymes into corresponding electrical signals with the aid of transducer.

- Now, the outcome from transducer i.e. electrical signal is a direct representation of the biological material (i.e. analyte and enzyme in this case) being measured.

The electrical signal is usually converted into physical display for its proper analysis and representation

Working principle of biosensors:

- The union of biological sensitive element and a transducer is responsible to convert the biological material into a corresponding electrical response in form of signal.
- The output of the transducer will be either current or voltage relying on the type of enzyme.
- If the output is voltage, then it is fine. But if the output is current, then this current needs to be converted into equivalent voltage (using an Op-Amp based current to voltage converter) before proceeding further.
- The output voltage signal is generally very low in amplitude and is superimposed on a high frequency noise signal.
- Thus, the signal is amplified (using an Op-Amp based Amplifier) and then it is passed through a Low Pass RC Filter.
- Signal Processing Unit or a Signal Conditioning Unit is accountable for performing the this process of amplifying and filtering the signal .
- The output of the signal processing unit is termed as an analog signal. This output is equivalent to the biological quantity being measured.
- The analog signal can be exhibited directly on an LCD display but usually, this analog signal is passed to a Microcontroller, where the analog signal is converted into digital signal. This is done since it is easy to analyse, process or store a digital signal.

Types:

- On the basis of sensor device as well as the biological material the biosensors are classified as:
    - 1. Electrochemical biosensors
    - 2. Calorimetric/Thermal detection biosensors
    - 3. Optical biosensors
    - 4. Piezo-electric biosensors
    - 5. Resonant biosensors

Electrochemical biosensors:

- Generally, electrochemical biosensor works on the principle that many enzyme catalysis reactions consumes or generates ions or electrons causing some change in electrical properties of the solution which can be detected and used as a measuring parameter.
- For example some biological compounds such as glucose, urea, cholesterol, etc.) are not electroactive, so the combination of reactions by this biosensor produce an electroactive element. This electroactive element results in change of current intensity which is proportional to the concentration of analyte.
- An electrochemical biosensor uses an electrochemical cell with electrodes of different dimension and modifications.
- Three kinds of electrodes are generally used-
    - Working electrode
    - Reference electrode
    - Counter or Auxilary electrode
- It is the working electrode where reaction occurs between electrode substrate and analyte.

Types of electrochemical biosensors

- Electrochemical biosensors are classified into three types:
    - **Amperometric Biosensors**
    - **Potentiometric Biosensors**
    - **Conductimetric Biosensors**
- **1. Amperometric Biosensors**
    - The Bioelectrochemical reaction in this biosensors generate measurable amount of current which is directly proportional to the substrate concentration.
    - The first generation amperometric biosensors use the Clark oxygen electrode which determines the reduction of O2 present in the analyte solution.
    - Determination of glucose using glucose oxidase enzyme is a redox reaction which is an example of Amperometric biosensors.
    - This first generation biosensors depend on the dissolved O2 to measure the concentration analyte. However, as modification in second generation biosensors, a mediators is being used.
    - This mediators transfer the electrons produced by the bioelectrochemical reaction directly to the electrode instead of reducing O2 dissolved in analyte solution.
    - Nowadays, the electrodes remove the electrons without the aid of mediators and are coated with electrically conducting organic salts.
- **2. Potentiometric biosensors:**

- Potentiometric biosensors use the ion-selective electrodes to convert the biological reaction to electronic response.
- Most commonly used electrodes are pH meter glass electrodes (for cations glass pH electrodes coated with a gas selective membrane for CO2, NH or H_2S.) or solid state electrodes.
- Biosensors detects and measures the ions or electrons generated in many reactions, very weak buffer solutions are used in this case.

- Gas sensing electrodes detect and measure the amount of gas produced**3. Conductimetric biosensors:**
  - These biosensors measure electrical conductance/ resistance of the solution.
  - Conductance measurement have comparatively low sensitivity.
  - Electrical field is generated by use of sinusoidal (ac) voltage which serves in reducing unwanted effects such as:
    - Faradaic processes
    - Double layer charging
    - Concentration polarization

2. Calorimetric/Thermal detection biosensors:

- Most of the enzyme catalysed reactions are exothermic in nature.
- Calorimetric biosensors measure the change in temperature of analyte solution following enzyme action and interpret it in terms of analyte concentration in the solution.
- The analyte solution is passed through a small packed bed column consisting immobilized enzyme.
- The temperature of the solution is measured just before the entry of the solution into the column, and just as it leaves the column using separate thermistors.
- It is the most usually applicable type of biosensor and can also be used for turbid and colourful solutions.
- There are demerits such as:
  - The biggest demerit is to maintain the temperature of the sample stream say + or -0.01°C.
  - Low range and sensitivity.

3. **Optical biosensors:**

- Both catalytic and affinity reactions are measured by this biosensor.
- The products generated during the catalytic reactions cause a change in fluorescence that is measured by the biosensor.

- In other way, biosensors measure the change induced in the intrinsic optical properties of the biosensor surface due to loading on it of di-electric molecules like protein.
- A most advanced biosensor involving luminescence uses luciferase enzyme for detection of bacteria in food or clinical samples.
- In the presence of O2, luciferase takes up the ATP released from the lysis of bacteria to produce light which is detected and measured by biosensor.

4. **Piezo-electric biosensors:**

- In these biosensors, the surface is coated with antibodies which binds to the complementary antigen present in the sample solution.
- This results in increased mass which decreases their vibrational frequency, this alteration/change is used to determine the amount of antigen present in the sample solution.

Resonant biosensors:

- The vibrations of the electron cloud in a molecule is termed as resonant biosensors.
- These plasmons oscillate at a particular frequency characteristic of the material.
- The oscillations in surface plasmons are confined to the surface of the material.
- Generally, gold or silver surfaces are preferred for the SPR based biosensors.
- When electromagnetic radiation falls on the metal surface, at a particular angle of incidence, the frequency of the electromagnetic radiation matches the frequency of vibrations resulting in resonance.
- The resonant angle depends on the refractive index of the medium.
- The refractive index in turn is determined by the local mass density on the metal surface.
- If the surface of the metal film is modified with the antibody/receptor i.e. capture molecule, then specific binding occurs between the capture molecule on addition of the sample and its ligand leading to an alteration in mass and hence change in resonant angle.
- These biosensors are employed to understand the functional aspects of human immune deficiency virus (HIV) both qualitatively and quantitatively.
- The major merits of these biosensors are rapid measurements and relatively high sensitivity,
- The major demerit is that it cannot be used to detect and measure the turbid and coloured solutions. In few cases, ligands may interfere with the binding.