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MBC-004: ENZYMES AND THEIR APPLICATIONS

Block

IV

PURIFICATION AND APPLICATION OF ENZYMES

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Purification of enzymes is of immense importance to obtain structural and functional information to determine their applications. The final degree of enzyme purification depends upon its end use. The key objective of the strategy for enzyme purification is to obtain the greatest possible yield of the desired enzyme with the highest catalytic activity and the greatest possible purity. Enzyme purification is a critical process in biochemical research, industrial applications, and pharmaceuticals. Different techniques such as precipitation, chromatography, and electrophoresis are used for the isolation and purification of enzymes. Most of the purification methods used in laboratory research can be easily extended to industrial processes.

Man has been using enzymes from several centuries to make several products such as cheese, yoghurt, bread, beer, and wine. The ability of enzymes to speed up the rate of reactions as well as enzyme specificity makes it special and attractive for industrialists. Since the 1950s, enzyme technology has really taken off. There are great benefits in using enzymes as catalysts to make products. Today the global market of enzymes is worth several billion dollars. In general the usage of enzyme is considered very safe, cost-effective and green or environment friendly technology. Industries extensively using enzymes are detergent industry, food & beverage, animal feed and pet food, detergent, textile, leather, pulp & paper and so on.

Since enzymes do not undergo any chemical change during the reaction to retain their catalytic activity, for commercial purposes the reuse of enzymes is possible only through immobilization. Latest innovations in immobilization technology have led to successful commercialization of immobilized enzymes. The cost of most industrial enzymes is often only a minor component in overall process. An immobilized catalyst offers several advantages for example, enabling continuous production, improved stability and the absence of the biocatalyst in the product stream.

Enzyme engineering has led to revolutionize enzyme technology by providing tailor-made enzymes for extended range of applications. Through several leading approaches such as directed evolution, rational design, and semi-rational design, enzymes with high efficiency and catalytic power can meet specific industrial, medical, and environmental needs. With the assistance of computational tools, high-throughput screening technologies, and extensive knowledge of enzyme structure-function relationships enzyme engineering has taken a huge leap.

Unit-10 of the block deals with different enzyme sources and the strategies to select a particular source so as to obtain maximum yield of the required enzyme with the highest catalytic activity and purity. Several physical and chemical methods of cell disruption for the extraction of enzymes have been discussed in the unit. Various separation methods used in the purification of enzymes have been dealt in the unit. Unit-11 deals with diagnostic enzymes. Unit-12 describes industrial application of enzymes in several industries such as starch, food, beverages-wine, fruit, leather, textile, detergent, paper and other industries. Unit-13 illustrates several different methods for enzyme immobilization and enzyme engineering.

Expected Learning Outcomes:

After studying this block, you should be able to:

- determine methods of extraction of enzymes from different sources,
- illustrate different steps to purify an enzyme,
- understand the role of diagnostic enzymes,
- explain the potential of enzymes in industrial processes and related commercial aspects,
- describe the role of enzyme in medicine,
- discuss the fundamental concept of immobilization, and
- enlist different ways of enzyme engineering and their applications in biotechnology and pharmaceutical industry.

UNIT 10

EXTRACTION AND ISOLATION OF ENZYMES

Structure

10.1 INTRODUCTION

Why should we isolate enzymes? The learner out of his/her curiosity might ask this question. The answer is enzymes work in a complex system of cell, tissues and organs. If we want a full and better understanding of enzyme, we need to isolate and purify them. Enzyme purification is a method of separating and isolating enzymes from other components of the cell to get enzymes in pure form with maximum possible yield. Several purification methods have been developed over the years taking into consideration the physico chemical properties of the enzyme of interest.

This chapter will include the detailed understanding of the nature of enzymes, different enzyme sources and the strategies to select a particular source so as to obtain maximum yield of the required enzyme with the highest catalytic

activity and purity. In the second part, we shall be discussing the principles and techniques involved in isolation and purification of enzyme. In the end, the enzyme purification procedures will be explained by giving suitable examples so as to give idea of the range of problems faced in this type of work.

The detailed studies demand a complete understanding of each and every aspect of enzyme from physical to catalytical, kinetics to control mechanism. Let us discuss them more in detail in the unit.

Expected Learning Outcomes

After studying this unit, you should be able to:

- describe the objectives and strategies of enzyme purification;
- explain different sources of enzyme;
- physical and chemical disruptive methods of enzyme isolation;
- separation methods of enzyme isolation; and
- discuss several steps of enzyme purification.

10.2 OBJECTIVE AND STARTEGY OF PURIFICATION

The aim and objectives of enzyme purification is:

a) isolation of enzyme with maximum possible yield,

b) isolation of enzyme with maximum catalytic activity,

c) enzyme should have maximum possible purity.

The catalytic activity of enzyme is determined by suitable assay procedure. The purity of enzyme is determined by specific enzyme activity under a given set of conditions.

The strategy for enzyme isolation and purification is also very important (Fig. 10.1). The general procedure of enzyme isolation and purification involves:

1) source of enzymes

2) enzyme solubilization methods and physical and chemical separation methods

3) purification methods

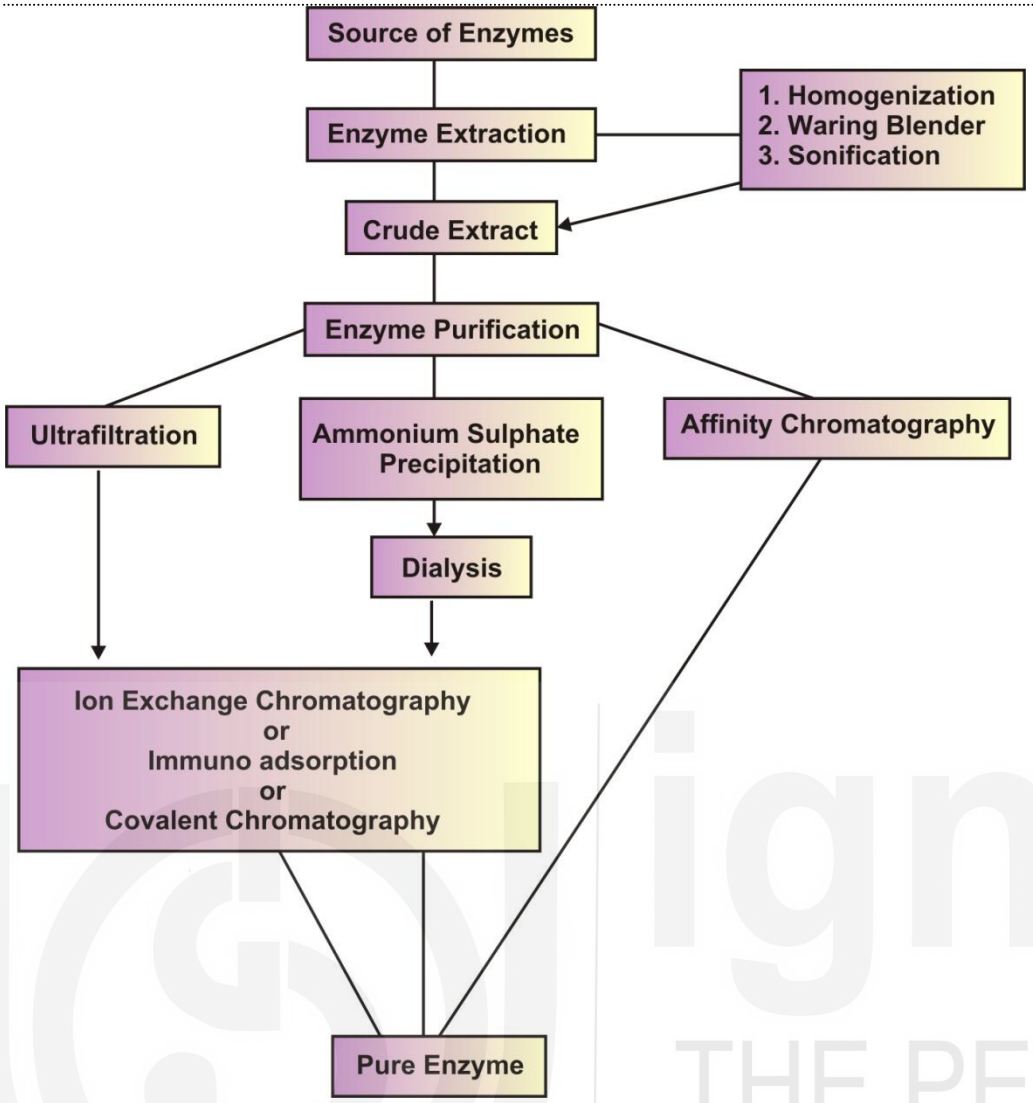


Fig. 10.1: Steps in the process of enzyme purification.

10.3 SOURCES OF ENZYMES

A number of factors influence the choice of starting material (raw material) in enzyme purification. These are:

- abundance of enzyme
- availability
- comparative studies
- subcellular location

10.3.1 Source and Abundance of Enzymes

The source as well as physical and chemical conditions of an enzyme significantly impacts its efficiency, purification process, stability, and cost. Historically, plant and animal materials were considered the best sources of enzymes due to their ease of extraction and cost-effectiveness. With animals, the principal species chosen are rat (for liver studies) rabbit (for muscle studies) and meat animals. Studies on human are limited due to limited availability of materials other than blood and placenta. However, as demand

for enzymes increased, animal sources became limited and expensive, while plant sources were influenced by weather and international politics. The change in quality during different seasons in plants can be overcome by growing them in special growth chambers. Plants cells are highly compartmented and bulk of volume in vacuoler space is filled with acidic solutions and proteases. These limitations along with advancements in fermentation technology, led to a rise in the popularity of microbially produced proteins. In recent years, animal and plant-derived enzymes have increased in cost, while microbial-derived enzymes have decreased.

The first and foremost choice for enzyme isolation involves choice of tissue to be used for purification. Firstly the tissue from which enzyme is to be isolated should be determined then the species from whose tissue will be taken should be chosen based on its ease of raising and growing. The classical method of enzyme purification involves selecting a source with abundant enzymes, such as lactating mammary gland is an excellent source of enzymes such as acetyl CoA carboxylase for fatty acid biosynthesis, and kidney as source for hydrolytic enzymes like alkaline phosphatase for phosphate esters hydrolysis at alkaline pH.

Prokaryotes are far better choice than eukaryotes for their offer as enzyme source because of their rapid growth and simple nutritional requirements. For eukaryotic proteins, lower eukaryotes such as yeasts are considered to be better as they show high growth rates on simple media. Prokaryotes and lower eukaryotes are better understood also at the genetic level. Therefore microorganisms and yeast are rapidly employed for enzyme production in fermenters. The media components, primarily waste materials and by-products from food and agriculture, provide carbohydrates, minerals, nitrogen and vitamins provides the requisite nutritional requirements for their growth and development. The quality and composition of these materials vary, affecting enzyme productivity. The chosen media is cost-effective and typically buffered at a pH for maximum stability.

Nowadays, researchers are also using recombinant DNA technology to increase enzyme production using microorganisms. This technique is used to create strains that produce excessive amounts of an enzyme. Successful expression occurs when the host organism is similar to or closely related to the gene being expressed. Gene over-expression is typically done by incorporating the gene in a plasmid under a strong promoter. However, bacteria are not ideal for expressing eukaryotic proteins due to their lack of post-translational machinery. Instead, lower eukaryotes like yeast are used due to their growth rates and genetic understanding. However, yeast strains like Kluyveromyces lactose and methylotroph Pichia pastoris are increasingly used. Baculovirus-driven expression in insect cells is also a preferred system for some applications, as it employs many of the protein modification, processing, and transport systems of higher eukaryotic cells.

10.3.2 Availability

High abundance of an enzyme does not always ensure easy availability. Several reasons can be attributed such as economic or geographical factors.

For instance, bioluminescent species are often found in exotic locations, and human or calf tissue is not widely available. This necessitates a compromise between abundance and availability, necessitating the development of suitable expression systems.

10.3.3 Comparative Studies

Researchers can study enzyme evolution by comparing a specific enzyme across different species or tissues. This can be beneficial in understanding the role of enzymes in different tissues, such as the properties of different isoenzymes of LDH in the heart and muscle.

10.3.4 Subcellular Location

Enzymes may be present in different fractions of cell or it may be localized to a particular cell fraction. Enzyme such as succinate dehydrogenase catalyze reactions in a single cell location, like mitochondria, allowing purification to homogenize or extract the entire tissue. However, in some cases, sub-cellular fractionation may be necessary, typically achieved through centrifugation steps, with larger organelles (nuclei and mitochondria) sedimenting first.

The best strategy except for few exceptions for enzyme separation will be to use the raw material as soon as possible because with the passage of time, natural degradative processes in dead source will set in. However, there could be several inherent limitations in using techniques and tools for enzyme separation immediately, in such a scenario; the enzyme extract can be frozen. Several commercial freezers are available with temperatures below -25^{o}C and -80^{o}C. However, there is possibility that on freezing ice crystals grow and could be highly destructive for membrane enzymes, salts can come out of solution thereby changing the pH before solidification set in. All these factors can harm the process of enzyme isolation and purification.

SAQ 1

a) Which one of the following is not a factor that limits the selection of right starting material for enzyme production?

   i) Source selection

   ii) Availability of enzyme in the source

   iii) Isolation procedures

   iv) Location of enzyme

b) The most preferred source of enzyme for large scale production are

   i) Plants

   ii) Microorganisms

   iii) Animals

c) Different isoenzymes help in understanding ……..of enzymes in different tissues.

d) These day ——— is being used to increase enzyme production in microorganisms.

10.4 CELL DISINTEGRATION AND EXTRACTION TECHNIQUES

Enzyme extraction requires cell disruption, so the least disruptive method should be used to release most enzyme, minimize extract contamination, and simplify purification. Enzyme extraction involves determination of the enzyme's cellular location, identification of intracellular enzymes within the cell, and extracellular enzymes, which are easier to isolate, found outside the cell.

- If it is **extracellular**, no extraction is necessary; for example, the pancreas in most mammals' produces a juice that contains a variety of enzymes (proteases, lipases, and esterases) which are synthesized and then excreted. Some of the well known examples of extracellular enzymes include lysozyme, ribonuclease and chymotrypsin. Many microorganisms also produce enzymes, which are excreted into the nutrient medium being fed to them. In such cases, isolation is very straightforward. One simply collects the fluids involved (an excreted juice or a nutrient medium) by filtration or centrifugation.
- For **intracellular** enzymes, the isolation and separation procedures are not so easy. The source of enzyme production may be an insect, fish, mammal, plant, or microorganism.

  The treatment of plant and animal enzymes is similar to extracellular enzymes, as they are soft and easily macerated. However, most microorganisms are mechanically robust and require more vigorous disruption methods to release their soluble intracellular enzymes. Depending on the characteristics of cells, the methods used for cellular disintegration are broadly divided into two types:

- physical disruption method,
- chemical disruption method.

10.4.1 Physical Disruption Method

Depending on the physiology of the organism, the energy used for the breakage of the cells varies.

a) Grinding with abrasive material: Small-scale production involves grinding with alumina or glass beads, disrupting tough microbial cells like glucose dehydrogenase from B subtilis. Large-scale production uses appliances like glass or metal heads in grinding mills, which can be mounted in a safety cabinet, allowing use with potentially pathogenic organisms.

b) **Liquid shear:** Liquid shear is a method used to disrupt bacterial, yeast, and fungal cells by passing cells through an orifice at high pressure into a chamber at atmospheric pressure. This method generates large amounts of heat, requiring efficient cooling. Larger scales have lower pressure drops, resulting in less disruption in a single pass. Liquid shear is useful for less robust organisms like gram-negative bacteria, but continuous recycling is needed for maximal disruption.

c) **Solid shear:** This method involves high-pressure extrusion of cell material, resulting in ice crystal formation, for small-scale production. It requires shear forces in heavy metal ions or air interfaces, and requires large energy to gather heat. Enzymes require cooling and may be stabilized by substrates, substrate analogues, or pollen.

d) **Ultrasonic cell disruption:** Ultrasonication, a method used in the chemical industry, involves the rapid sinusoidal movement of a probe within a liquid, causing microbial cell inactivation and disruption.

e) **Use of bead mills:** Cell suspensions agitated with small beads can be broken by high liquid shear gradients, affecting enzyme release rate and effectiveness. Adjustments in agitation rates, bead size, and equipment dimensions can modify this process.

f) **Use of freeze- presses:** The Hughes and 'X' presses force frozen cell pastes under high pressure, causing disruption due to phase and volume changes and solid shear from ice crystals, with factors controlling cell breakage.

g) **Freeze- thaw:** The technique is useful with certain susceptible microbes and eukaryotic cells where repeated freezing and thawing results in extensive membrane lesions with release of periplasmic and intracellular proteins.

10.4.2 Chemical Treatment Method

These include lytic methods, where cell breakage is done by using non-mechanical methods to get the enzymes released under gentle conditions. The available methods include:

a) **Treatment with alkali:** The enzyme L-asparaginase from Ervinia can be produced at a pH range of 10.5-12.5 using NaOH, a simple and inexpensive method that remains stable for 20-30 minutes during treatment. This method has been applied to the isolation of one enzyme on a large scale.

b) **Detergents:** Detergents, such as chelates, sodium dodecyl sulphate, CTAB, tween, and triton, are used to extract membrane-bound enzymes like cholesterol oxidase from Nocardia sp. However, these detergents can denature most enzymes, making them less preferable than non-ionic detergents. Additionally, gas-liquid phase interfaces in foams can disrupt enzyme conformation.

c) **Osmotic shock:** This method is suitable for gram-negative organisms due to osmotic shock, which releases hydrolytic enzymes in periplasmic spaces, but many organisms are resistant to this shock.

d) **Organic solvents:** Organic solvents like isopropyl alcohol, ethanol, and ethyl acetate are used for extracting enzymes from yeast cells, but are toxic, costly, and can cause protein denaturation and flammability.

e) **Lysozyme and EDTA:** Lysozyme is a specific method for breaking down gram-positive bacteria's cell walls, but its high cost makes it unsuitable for large-scale production. In plant tissue culture, lytic enzymes are used to prepare protoplast.

f) **Acetone powder:** Acetone drying is an effective method for breaking cell membranes and preparing tissue powder for long-term storage in a deep freezer, but requires low temperature precautions to avoid enzyme denaturation.

All of these procedures resulted in the cell membrane being ruptured and the intracellular components being released into the surrounding medium.

Optimization and Clarification of extract

Since the properties of proteins depends not only in their own particular characteristics but also on the composition of solution. Large-scale enzyme extraction face unique challenges compared to laboratory methods, necessitating a comprehensive understanding of the entire process before optimising any individual step. Several things can go wrong while using different extraction methods from bead mill to waring blender, the extract will become more dilute and with a different composition, several components may get disrupted. It is often very necessary to cool the system during disruption; ice cold buffers and short duration of treatments followed by a cooling span to prevent the protein from getting denatured or lost during the process. It is always desired to measure the enzyme specific activity at each step of procedure.

It is preferable to add β-mercaptoethanol in the extractant buffer. Cells contain many salts and charged insoluble material such as proteins, phospholipids and nucleic acids. To ensure that all soluble cell constituents are extracted, it is always preferable to use a buffer of ionic strength similar to physiological one with appropriate pH. Typical buffers would be 20-50 mM phosphate, pH 7.0-7.5; 0.1 M Tris-HCl, pH 7.5 with inclusions of EDTA (1-5 mM), cysteine 95-20mM) with specific stabilizing agents or cofactors. Several times, the extract becomes turbid due to fat particles floating on the surface. These particles can be removed by filtration through a plug of glass wool or a fine mesh cloth. Clarification of the extract can be carried out by membrane ultrafiltration. Ribsosomal and other nucleoproteins can be removed by acidification through isoelectric precipitation. While working with microorganisms; large amount of nucleic acid material from cells can cause considerable problems. So a variety of substances can be used such as streptomycin, protamine and polyethyleneimine can be used to cause precipitation of nucleic acids and associated compounds but their high cost discourages their use. Enzymic

digestion with nucleases is easy and cheap, but does not remove nucleic acids but shortens them, reducing solution viscosity. For the extraction of membrane proteins, a combination of following factors will be helpful to isolate the desired protein/enzyme. These factors are:

a) Metal chelators EDTA or EGTA 1-10mM

b) Mild alkaline conditions (pH 8-9) at low ionic strength

c) Dilute non-ionic detergent (Tween, Triton X-100, Digitonin etc)

d) Low concentrations of organic solvents such as n-butanol

e) High ionic strength e.g 1 M NaCI

f) Phospholipase treatment

After using any of the above mentioned physical or chemical methods, the next process involves centrifugation or filtration to separate cell debris, yielding a clear extract with potential nucleic acids, salts, nutrients, and carbohydrates. Preliminary purification and concentration steps are followed, resulting in a concentrated enzyme.

SAQ 2

a) Enzymes which are secreted outside the body cells are referred to as …… enzymes.

   i) extracellular

   ii) intracellular

   iii) cytoplasmic

   iv) mitochondrial

b) Which of the following is not a technique for the extraction of enzymes by chemical methods?

   i) Alkali treatment

   ii) Osmotic shock

   iii) Detergent treatment

   iv) Homogenisation

c) In _____ treatment, produced basic radicals mediate a nucleophilic attack on compounds of cell wall on membrane leading to destabilization and fragmentation of membrane followed by release of enzymes.

d) Homogenization utilising _____ is one where in manual pressure is created for cell lysis.

10.5 METHODS FOR ENZYME PURIFICATION

The purification of an enzyme involves removal of other substances, including other undesired proteins and non-proteins, present in the preparation. Enzyme can be purified several hundredfold but the yield of the enzyme may be very poor, frequently below 10% of the activity of the original material.

10.5.1 Purification Methods

Enzyme purification is a multi-step process which has to take into account about the biophysical and biochemical characteristics of enzyme. The design should be reproducible as well as economical. It should focus on high yield and recovery of enzyme making economical use of reagents and chemicals, and shorter purification time. Enzymes have unique physico-chemical characteristics; the chosen purification technique should be moderate and tend to maintain the enzyme's native conformation without any changes.

Enzymes are more stable in dilute solutions, so purification procedures initially concentrate the enzyme protein rather than focus on purification to minimize loss of the enzyme activity.

You have already read about most of the techniques listed below in your MBC-003 course on Bionanalytical Tools and Techniques. In this unit we will discuss their employability with reference to enzyme isolation and purification. The main properties of enzymes that can be exploited in separation methods are listed in Table 10.1:

Table 10.1: Separation methods used in the purification of enzymes

Property	Method	Scale
Size or mass	Centrifugation Gel filtration Dialysis, Ultrafiltration	Large or small Generally small Generally small
Polarity A) Charge B) Hydrophobic character	Ion exchange chromatography Chromatofocussing Electrophoresis Isoelectric focussing Hydrophobic chromatography	Large or small Generally small Generally small Generally small Generally small Generally small
Solubility	Change in pH Change in ionic strength Decrease in dielectric constant	Generally large Large or small

		Generally large
Specific binding sites or structural features	Affinity chromatography Immobilised metal ion chromatography Affinity elution Dye-ligand chromatography Immunoadsorption Covalent chromatography	Generally small Generally small Large or small Large or small Generally small Generally small

Methods based on size or mass

a) **Centrifugation:** Centrifugation is the preferred method for collecting enzyme-containing solids due to its safety and minimal foam production. It separates material based on particle size and density, and large molecules like enzymes can be sedimented using high centrifugal fields from an ultracentrifuge, with sedimentation rate increasing with higher Mr value. Generally centrifugation is extensively used to remove the precipitated material in the process of enzyme isolation eg. removal of cell debris after homogenization or to collect the precipitated enzyme by addition of ammonium sulfate.

However, in order to obtain pure preparations, the sedimented fractions are usually resuspended in buffer and then re-sedimented once or twice more.

b) **Flocculation:** High-molecular-weight charged materials create a loose aggregate that can be removed through centrifugation or filtration. Flocculation and coagulation are cost-effective aids for precipitating cells, debris, or proteins, but must not inhibit target enzymes.

c) **Gel filtration:** Gel filtration is a column chromatographic procedure used for fractionating high-molecular-weight materials based on their molecular size. It separates molecules based on their ability to enter the pores within the gel beads. Small molecules are retarded as they pass down the column, while large molecules are eluted first. The most widely used gels are Sephadex, Bio-Gel P, Sephacryl, and Sepharose. The degree of cross-linking controls the size of the pores, allowing for a variation in the range of Mr values that can undergo fractionation. Gel filtration can be performed on a large scale, but is more suitable for small-scale enzyme purification stages due to time and cost.

d) **Filtration:** Large-scale filtration uses filter cloths and aids in rotary vacuum or centrifugal filters, but efficiency is limited by particle shape and compressibility.

d) **Dialysis and Ultrafiltration:** Dialysis is a process to remove low molecular weight contaminants and salts. The dialysis membrane's ability to allow free flow of components, its relative surface area to the solution volume, and the concentration of diffusable substances outside the membrane are key factors. Common dialysis membranes include animal membranes, collodion, colloids deposited in porous pots, and cellophane tubing. These membranes create a molecular sieve, allowing low molecular weight substances to pass while retarding high molecular weight proteins.

A dialysis membrane, like cellophane, acts as a sieve for globular proteins with Mr values up to 20,000, but not large molecules. It's not ideal for enzyme separation but is used in purification to remove salt, organic solvents, or low Mr inhibitors from enzyme solutions. An alternative method is centrifuge tubes, which have two compartments separated by a membrane, allowing small molecules to pass through. Semipermeable membranes are primarily used for large-scale enzyme purification in the latter stages, primarily for concentration and salt removal, making dialysis impractical due to the requirement of large volumes of buffer/distilled water.

Ultrafiltration is a preferred method for purifying samples, as it removes water and low-molecular-weight materials through a membrane under pressure, while retaining enzymes. It involves feeding a protein sample into a cell with a membrane, which retains high molecular weight proteins while filtering solvent and low molecular weight molecules. Nitrogen gas is used to maintain flow across the membrane. Smaller pore size membranes are available for retaining and concentrating low molecular weight proteins and for enzyme purification. This process reduces sample volume and is different from conventional filtration and microfiltration in retaining particles smaller than 50nm in diameter.

Methods based on polarity

a) **Ion exchange chromatography:** Ion exchange chromatography is a method used for purifying enzymes by applying them to an ion exchanger in a low-ionic-strength solution with opposite charges. Desorption of bound species can be achieved by changing the pH or increasing the ionic strength of the solution. This gradient allows for protein separation based on their ability to bind to the ion exchanger. However, pH changes have not been effective in enzyme purification due to the buffering properties of bound enzymes. Chromatofocussing, a technique that uses high-buffering ampholytes, overcomes these issues by creating a pH gradient on the column. The protein mixture adsorbed on the ion exchanger at the upper end of the pH range, and the pH gradient generated by the addition of the acid form of the ampholyte buffer generates the gradient. This technique has high resolving power but is expensive for large-scale purification procedures due to the high cost of materials involved.

Ion exchange chromatography can be performed on large or small scales. Large scales often work batch-wise, absorbing enzymes and

pouring them into a column for controlled desorption. Small scales involve both adsorption and desorption in a column.

Ion exchange chromatography can achieves up to 10-fold purification, with high-performance ion exchangers like monoQ being widely used in modern purification procedures, enhancing the efficiency of the process.

b) **Electrophoresis:** Electrophoretic separation involves the movement of charged molecules under an electric field in a medium like paper, cellulose powder, starch, or polyacrylamide gels. The enzyme's position is determined using Coomassie blue or silver stains. This technique can be used on a small analytical scale or large preparative scale, and the separated enzymes can be eluted.

Capillary electrophoresis is used for separating and analysing enzyme mixtures in a capillary tube, allowing temperature control. The tube is filled with buffer and the sample is introduced by applying an electric field. The components of the mixture are detected as they pass through a window at the far end.

c) **Isoelectric focussing:** Isoelectric focussing is a method used to determine the equilibrium position of charged species in a pH gradient. It involves applying a potential gradient to a gel containing a mixture of ampholytes (polyamino acids with different charge properties). When the potential difference is applied, negatively charged molecules migrate towards the anode, while a positively charged species move towards the anode. This results in a pH gradient, allowing an enzyme to migrate to the position where the gel's pH equals its isoelectric point. This method is useful in purification procedures and can be scaled up for preparative work.

d) **Hydrophobic interaction chromatography:** Hydrocarbons and non-polar molecules associate with each other in equilibrium environments. Protein surfaces contain charged and polar amino acid side chains, which enable them to bind to non-polar molecules and cause aggregation. Hydrophobic interaction chromatography uses these interactions to adsorb proteins on matrices like octyl- or phenyl-sepharose at high ionic strength. Desorption of proteins can be achieved by decreasing ionic strength and adding organic solvents or non-ionic detergents.

Methods based on changes in solubility

Solubility of a compound in a solvent depends on the balance of forces between solutes, and solute and solvent. Insoluble compounds are insoluble if the former dominates, while soluble compounds are soluble if the latter dominates. Enzyme purification process makes use of this fragile balance to obtain desired enzyme precipitates or to remove contaminants.

There are three important ways of changing the stability of enzymes:

- to change the pH,

- to change the ionic strength, and

- to decrease the dielectric constant.

a) **Change in pH:** At its isoelectric point, enzyme is the least soluble, as it lacks repulsive electrostatic forces. Adjusting the pH can precipitate an enzyme, but it's crucial to ensure enzymes aren't inactivated by these changes.

b) **Change in ionic strength:** Large charged molecules are faintly soluble in pure water, the addition of ions encourages solubility by disbanding the charge carried by large molecule. The process is known as "*salting in*". However, if ionic strength increases beyond a certain limit, the charged molecule will precipitate (*salting out)*. It is argued that high concentrations of salt decrease water concentration, leading to decreased solute-solvent interactions and hence solubility. This process removes non-protein impurities in enzyme homogenate. Solid salt concentrations range from 0 to 90%, with gentle stirring. The suspension is stored in cold conditions for precipitation, then collected generally after centrifugation and further dissoluted in a suitable medium. The protein solution is desalted using dialysis or Sephadex G-25 gel filtration chromatography. Ammonium sulphate is the preferred salt due to its low cost, high solubility, and lack of harmful effects on most enzymes. However, precipitation due to its usage poses handling and disposal issues due to its high concentration, which can corrode concrete and stainless steel. Other salts like sodium sulphate can also be used but requires temperatures above 35ºC for fractional precipitation. Enzymes typically precipitate at a certain concentration, forming the basis of initial fractionation procedures. Nonionic polymers like polyethylene glycol (PEG) are non-toxic and non-flammable but restricted due to their high viscosity. Precipitation can be negative or positive, with the latter being preferred as it can be redissolved in minimal volume. In some cases, pure enzyme can be obtained through ammonium sulphate fractionation of crude extracts, like fructose-bisphostphate aldolase from rabbit muscle.

c) **Decrease in dielectric constant:** The addition of a water-miscible organic solvent, like ethanol or acetone, decreases a solution's dielectric constant, increasing electrostatic forces and precipitating large, charged molecules like enzymes. This method is suitable for large-scale fractionation, but can sometimes cause enzyme inactivation, so low temperatures are crucial for minimizing the inactivation.

Protein precipitation can be achieved by adding a neutral water-solube polymer, poly (ethylene glycol), which removes water from protein hydration spheres, resulting in successful purifications. However, removing residual poly (ethylene glycol) can be challenging.

All these methods discussed above can be applied on a large scale and are often used in the initial stages of purification.

Methods based on the possession of specific binding sites

Enzymes exhibit highly specific interactions with substrates and ligands, which can be advantageous in affinity separation methods during enzyme purification.

a) **Affinity chromatography:** Affinity chromatography is a method where an enzyme is linked to an inert matrix, like agarose, and only retained when passed down a column. This method can be desorbed by substrate pulses or changes in solution pH or ionic strength, allowing for the purification of an enzyme from crude extract in a single step.

There are, however, a number of problems associated with the use of affinity chromatography in enzyme purifications :

- attaching a suitable substrate analogue or inhibitor to the matrix can be a difficult task and reactions involved in coupling are not always completely characterized,

- linking of the ligand to the matrix may obstruct with the binding to the enzyme and lead to a loss of specificity in the interaction, and

- special problems are faced by enzymes that catalyze reactions involving more than one substrate.

Affinity chromatography, despite its high resolution and one or two-step purification processes, is not widely used in large-scale operations due to its expense, low capacity, and instability. In spite of these problems, affinity chromatography has made a significant contribution to the purification of enzymes as it is highly useful for low volume, adequate purity, and high-priced products.

b) **Affinity elution:** Affinity elution is a technique that combines affinity chromatography and desorption, with specificity occurring at the adsorption stage. It involves adsorption of desired enzymes onto an ion exchanger, like DEAE-cellulose, and elution by the appropriate substrate. The technique offer better advantage than affinity chromatography on the non requirement of employing complex task of designing and attaching ligand to matrix.

c) **Dye ligand chromatography:** A tandem strategy is employed for purification, where a crude extract is passed through a dye column that doesn't retain the protein of interest, removing contaminating proteins that might interfere with the second step where dye column retain the protein of interst. Cibacron Blue F3G-A dye binds to dehydrogenases and kinases, which share a nucleotide-binding domain structure. Although the specificity of this interaction is not fully understood, it has proven beneficial in purification processes.

Procyon Red HE-2B is a dye that binds to NADP+-dependent dehydrogenases, preferring gluose-6-phosphate dehydrogenase over malate dehydrogenase, making it useful for purifying crude yeast extracts, while Cibacron-Blue F3G-A binds NAD+-dependent dehydrogenases. Adsorption and elution conditions can vary, with

immobilised dyes acting as effective cation exchangers under certain conditions, requiring ionic strength > 0.1M and pH> 7.

d) **Immunoadsorption chromatography:** High specificity in antigen-antibody interactions can be used for purification procedures. A small amount of a pure enzyme from one species can be used to raise antibodies in another species, producing a range of antibodies with varying affinity for the enzyme. After purification, antibodies can be coupled to a matrix like CNBr-activated sepharose to separate the enzyme. Desorption can be achieved through changes in pH, ionic strength, or other treatments, which can destroy enzyme activity.

Monoclonal antibody techniques have been used to overcome problems by selecting antibodies with appropriate affinity for the enzyme antigen. Example includes aromatic L-amino acid decarboxylase from the bovine brain which can be purified using an immobilized antibody on an activated cross-linked agarose matrix. The enzyme can be eluted from the immobilized antibody using a solution containing 50 mmoldm-3 acetic acid and 10% ethylene glycol.

e) **Covalent chromatography:** This technique creates a covalent bond between the molecules in the mobile phase and the stationary phase matrix. Activated thiol-sephahrose 4B is a matrix used for purifying cysteine-containing proteins, such as papain, to separate a mixture of cysteine-containing proteins. It can be used to immobilize cysteine-containing proteins for affinity chromatography, such as cytochrome c oxidase, and can be used to separate protein-disulphide isomerase from glutathione-insulin transhydrogenase.

SAQ 3

a) Which of the following is not a step involved in purification?

i) Precipitation

ii) Dialysis or ultrafiltration

iii) Chromatographic techniques

iv) Freezing technique

b) ……. retains analyte molecules based on the coulombic interaction.

i) Thin layer chromatography

ii) Affinity chromatography

iii) Gel filtration chromatography

iv) Ion exchange chromatography

c) When the enzyme solubility is reduced by reducing the water availability, the enzyme-enzyme interactions start to take place and they will

aggregate to form precipitates. This phenomenon is called ……..… .

d) ……. is a divalent chelator which can be used to extract enzymes from different sources.

Other methods of purification

For large-scale production of enzymes at low cost, it may be advantageous to consider the production and purification of more than one enzyme from one process. Enzyme purification is a complex process, and various steps are to be conducted in sequence for the purification of enzyme. We have discussed several methods in previous sections, Let us discuss some of the other methods for enzyme purification.

a) Adsorption: Fractional adsorption is a method used to remove non-protein contaminants from enzyme preparations. It is useful in the initial stages of purification and concentration. Adsorbents like resins, substituted dextrans or agarose, and substituted cellulose can be used on a small scale, but are costly on a large scale. Materials like celite and hydroxyapatite are used due to their low cost and ability to differentially elution bound proteins. Adsorption techniques often involve elution, with the elution process being more efficient by dividing the effluent into lots. This approach is widely accepted in enzyme isolation and purification.

b) Aqueous two phase separations: This method is mainly done for the removal of polysaccharides and cell debris. This is a relatively recent introduction to purification technique. It has two main advantages-

-easy separation of proteins from bacterial cell walls and semisoluble debris, and

-its conversion to a continuous process.

The technique involves placing different proteins in a solution of two immiscible polymers, which separate into distinct phases. This selective adsorption of proteins occurs due to the physical nature of the molecules involved, as identical or similar molecules tend to aggregate more. For example, aqueous two-phase systems can be prepared by mixing polyethylene glycol and dextran or polyethylene glycol and specific salts. The enzyme is found in the upper polyethylene glycol-rich phase and the lower phosphate-rich phase. The separation process is carried out by centrifugation. This method is advantageous for large-scale enzyme purification.

c) Column chromatography: Chromatography is a protein separation technique that can be modified by changing packing chemistry and elution buffer. Older chromatographic materials are being replaced by materials like cross-linked celluloses, agaroses, polyacrylamide/agarose copolymers, and composite inorganic/cellulose materials. Commercially available fully automatic liquid chromatography equipment offers speed,

resolution, sensitivity, reproducibility, and recovery. Ion exchange chromatography, adsorption chromatography, gel filtration chromatography, and affinity chromatography are commonly used chromatography techniques for enzyme purification.

The procedure for purifying enzyme samples in chromatography involves applying the sample onto a pre-equilibrated column, eluting it with buffer, a gradient of solute, or a specific ligand, and collecting the effluent as fractions using a fraction collector. These fractions are then tested for enzyme activity.

d) **Chromatofocussing:** Chromatofocussing creates a pH gradient on an ion exchanger by buffering charged groups. This gradient is formed by mixing two buffers of different pHs using a linear gradient maker. This process results in band sharpening, sample concentration, and good protein resolution. Linear pH gradients are better for protein resolution. Commercial amphoteric buffers and poly buffer exchangers are available in different pH ranges.

f) **Continuous electrophoresis:** Electrophoretic techniques are no longer widely used for enzyme purification today. Continuous electrophoresis is a technique that uses an electric potential to apply a current across a protein solution, preventing sideways mixing. It involves two electrodes, a solid cathode and a rotating anode, which pass solvent between them. The outer electrode swirls the solvent around the inner electrode, stabilizing the flow. Despite its infancy due to expensive hardware, it is currently used for blood protein fractionation.

g) **Concentration and packaging:** Crystallisation is a process used to concentrate an enzyme after purification. It involves adding ammonium sulphate or salts like sodium sulphate to the concentrated enzyme sample, causing slight turbidity. After storage for 24 to 48 hours, crystals may appear. Crystallisation is not a proof of purity and may contain other proteins. Re-crystallisation increases the specific activity of the crystals.

Enzymes are packaged in various forms, such as lyophilised powders or suspensions in ammonium sulphate, depending on their purpose. For analytical or small-scale investigative work, they are usually packaged as a lyophilised powder or a concentrated solution. For bulk requirements, dust-free packaging is necessary, and enzymes can be combined with salts and waxes to create pellets or concentrated solutions. Evaporation can be used for complete drying when freeze drying is unpredictable due to enzyme scale or instability, and heat denatures proteins only if applied for a long period.

SAQ 4

a) Aqueous two-phase systems can be prepared by mixing …… and ..... (Fill in the Blanks).

b) Which technique is currently used for blood protein fractionation?

c) Name two types of column chromatography.

d) Crystallisation is a process used to concentrate an enzyme after purification (True/False).

10.6 CHOICE OF METHODS FOR ENZYME PURIFICATION

There is rarely only one method or combination of methods that can be used to purify a given enzyme. The actual sequence of methods employed will depend on a variety of sections such as:

- the scale of preparation and the yield of enzyme required,

- the time available for the preparation, and

- the equipment and expertise available in the laboratory.

Methods based on changes in solubility, such as pH, ionic strength, and dielectric constant, are more suitable for large-scale purification stages, while column chromatography or electrophoresis are more suitable for smaller stages. If proteolysis is a concern, quick purification may be preferred, with solubility-based methods being more efficient. Matrices, such as sepharose and other beaded hydrophilic matrices, can expedite column chromatography under HPLC conditions. However, these matrices are expensive and require specialized apparatus, making them generally used in the final stages of purification procedures.

Despite advancements in affinity techniques, many enzymes are still easily prepared using traditional methods like precipitation by ethanol or ammonium sulphate. Using new procedures like affinity chromatography may not be worth it unless there is evidence of heterogeneity or partial inactivation.

Exercise of protein purification method

Bromelian, a proteinase, can be extracted from pineapple (Ananas colossus) using different techniques including centrifugation, ammonium sulphate precipitation, dialysis and ion exchange chromatography. Learners should try to understand the each and every detail of the whole process of enzyme purification.

Homogenization: The fruit stem is first washed with 0.1% hydrogen peroxide solution, peeled, and cut into small pieces. These cut pieces are then homogenised in sodium acetate buffer solution and filtered to form the "crude extract."

Centrifugation: The crude extract is further centrifuged in a cooling centrifuge maintained at 4ºC at different speeds (i.e., 2000, 4000, and 6000 rpm) consecutively for 10 minutes each to collect the supernatant.

Salting-out: The first step in the purification process involves ammonium sulphate precipitation, or “salting-out," during which the salt is added to the supernatant pinch by pinch on continuous stirring with a magnetic agitator,

and incubation is done overnight at 4ºC. The precipitated enzyme is then resuspended in 10mM Tris HCl buffer.

Dialysis: The solution obtained from the salting-out process is transferred to a dialysis container and then agitated in a beaker containing 100mM phosphate buffer-NaCl solution overnight. The whole assembly is kept in a cool environment.

Ion exchange chromatography: The chromatography column is filled with DEAE cellulose and then equilibrated with a 0.5M sodium phosphate buffer (pH 8.0). Eluting buffer (containing 25mM Tris HCl and 25mM NaCl) should be added, and the dialysed bromelian sample will then be introduced carefully on the column. The enzyme gets eluted first using the initial eluting buffer, and the procedure should be repeated with different buffer solutions containing various concentrations of Tris HCl and NaCl. The eluents thus obtained using different buffer solutions should be evaluated for their activity.

Gel electrophoresis of isolated enzyme: The enzyme is loaded onto a 3.5% stacking gel, and electrophoresed onto a 12% separating gel at 200V using SDS-PAGE technique and the photograph showing bands should be obtained.

10.7 SUMMARY

In this unit, an overview of the wide range of techniques and procedures involved in the enzyme purifications has been given. Let us summarize them:

- The purification procedures that have been used to isolate specific enzymes from crude cellular extract containing many unwanted components. The extraction procedures involve the use of different extraction solvents to disrupt cells or tissues.
- A number of factors influence the choice of starting material (raw material) in enzyme purification such as abundance of enzyme, its availability and subcellular location.
- Enzyme extraction involves determination of the enzyme's cellular location, identification of intracellular enzymes within the cell, and extracellular enzymes, which are easier to isolate, found outside the cell.
- The methods used for cellular disintegration for enzyme extraction are broadly divided into two types: physical disruption method and chemical disruption methods.
- Differences in charge, solubility and size affect the separating procedure to be followed for isolating an enzyme of interest.
- Centrifugation is the extensive considered method for collecting enzyme-containing solids due to its safety and minimal foam production. It separates material based on particle size and density, and large molecules like enzymes can be sedimented using high centrifugal fields.
- Dialysis and Ultrafiltration are the vital processes to remove low molecular weight contaminants and salts.

- Ion exchange chromatography, Electrophoresis and isoelectric focusing and Hydrophobic interaction chromatography are some of the common methods based on polarity that are adopted for protein purification.
- The stability of enzyme can be altered by the change the pH or ionic strength or the dielectric constant. The changes can be used for the separation and purification of enzymes.
- Affinity chromatography, affinity elution, dye ligand chromatography, immunoadsorbtion chromatography and covalent chromatography are the different methods based on the possession of specific binding sites can be employed for enzyme purification.
- Adsorbtion, aqueous two phase separations, column chromatography, chromatofocussing and continuous electrophoresis are some of the other methods that can be used for enzyme purification.
- The targeted methods employability depends on the physical and chemical properties of the enzyme under investigation and also on the source from which the target enzyme is extracted.
- These procedures of isolation and purification of enzyme helps in studying the structure and function of enzyme, which can help in the development of new enzymes with improved properties.

10.8 TERMINAL QUESTIONS

1. How does the availability of enzymes affect the choice of enzyme sources?
2. Explain various physical disruption method for enzyme isolation.
3. Write a note on chromatofocussing.
4. What is meant by salting out phenomenon?

10.9 ANSWERS

Self Assessment Questions

1. a) Option (i), b) Option (ii), c) role, d) recombinant DNA technology
2. a) Option (i), b) Option (iv), c) alkali, d) mortar and pestle
3. a) Option (iv), b) Option (iv), c) salting out, d) EDTA
4. a) Polyethylene glycol and dextran, b) Continuous electrophoresis, c) Ion exchange, Gel filteration, d) True

Terminal Questions

1. Refer to section 10.1.2
2. Refer to section 10.3.1
3. Refer to section 10.4.1.2

4. Salting out is the phenomenon observed when the solubility of a non-electrolyte compound in water decreases with an increase in the concentration of a salt. Salting out results in the lower solubility of high molecular weight compounds in water. Salting-out extraction can also improve the recoveries in liquid-liquid extractions and create a phase separation between water-miscible organic solvents and water, making it advantageous for sample preparation techniques in analytical chemistry. Ammonium sulphate, $(NH_4)_2SO_4$, is often used for salting out because of its high solubility, which allows for solutions of very high ionic strength, low price, and availability of pure material.

10.10 FURTHER READINGS

1. Prasad NK. Enzyme Technology. Pacemaker of Biotechnology. 2011. PHI Learning Pvt. Ltd.
2. Singh S. A Textbook of Enzymes. 2007. Campus Books.
3. Grewal S, Mutha P. Enzyme Technology. 2010. Agrobios (Indoa).
4. Palmer T. Enzymes: Biochemistry, Biotechnology and Clinical Chemistry. 2004. East-West Press Put. Ltd, New Delhi.
5. Chauhan BS. Comprehensive Enzyme Technology. 2005. Satya Prakashan, New Delhi.
6. Price NC, Stevens L. Fundamentals of Enzymology. The Cell and Molecular Biology of Catalytic Proteins. 2010. 3rd Edition. Oxford University Press.
7. Dubey RC. Advanced Biotechnology. 2014. S Hand & Company Pvt. Ltd.
8. Pathak A. Tools and Techniques in Radiation Biophysics. 2023. Springer Publisher.

UNIT 11

DIAGNOSTIC ENZYME

Structure

11.1 INTRODUCTION

In the last unit you come across several techniques for the isolation and purification of enzymes. Purified enzymes have lot of applications in the industry and diagnostics is one of them. Enzyme diagnostics is one of the branches of enzymology. In this unit we will discuss about the different applications of enzymes as well as diagnostic enzymes and their related aspects. Enzymes are considered to be valuable markers in different stages of diseases and provide insights into the metabolic and pathological processes during the course of disease. As you know, these enzymes are vital indicators of cellular damage and have diverse applications in diagnostics, including immunoassays and biosensors. Their ability to detect specific conditions makes them valuable in diagnostic methodologies. In the clinical field, the enzymes perform a variety of roles, including treatment of enzyme deficiencies, diagnosis of diseases, managing wounds, and conducting analytical tests. They are also useful in detecting cellular disorders and tissue damage based on their levels or activity in biological fluids. Enzymes are used as markers for various diseases, like cancer, heart attacks, jaundice, pancreatitis, and neurodegenerative disorders. Furthermore, enzyme-based biosensors are employed in monitoring several diseases, drug discovery, and in detecting pollutants, disease-causing microorganisms, and disease indicators in fluids like blood, urine, saliva, and sweat. Enzymes are also among the most widely used biomolecules in research laboratories. Let us discuss the some of the applications of multifaceted enzymes.

Expected Learning Outcomes

After studying this unit, you should be able to:

- describe the application of enzymes in diagnostics;
- uses of enzymes as analytical agents;
- discuss the application of enzymes in medicine, agriculture and research;
- describe about enzyme therapy, and
- explain the role of enzymes as biosensors.

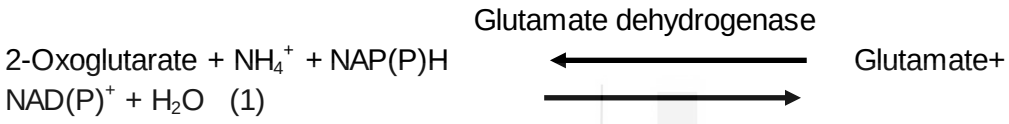
11.2 APPLICATIONS OF ENZYMES IN DIAGNOSTICS

Enzymes are utilized in diagnostic applications such as immunoassays and biosensors. These applications are categorized as enzymatic diagnostics, immunodiagnostics, and DNA diagnostics.

1. **Enzymatic diagnostics:** In your visit to the hospital, you may come across biochemical tests prescribed by the physician for the determination of disease or its progression. Many enzymes present in plasma or serum are routinely tested in clinical labs as these enzymes are of great diagnostic value. Each plasma enzyme has a normal

concentration range due to the balance that is set up between its rate of arrival and its rate of removal in the blood stream. These enzymes have a normal range of activity. If a particular tissue is affected by disease or gets damaged, some of enzymes may leak into the bloodstream at an increased rate and will be found in the plasma in elevated amounts. The detection and measurement of enzymes using biochemical methods helps in detection of disease via their correlation with the symptoms, progression of disease and even cure from the disease.

Diagnostic enzymes are crucial for disease diagnosis and monitoring using medical samples like urine, serum, and other body fluids. The enzymatic reactions are measured either by the rate of substrate consumption or the rate of product formation. For instance, glutamate can be estimated using NADH produced in a glutamate dehydrogenase reaction (equation 1).



Glucose can also be measured by a coupled enzymatic reaction of hexokinase (equation 2) and glucose-6-phosphate dehydrogenase (equation 3).

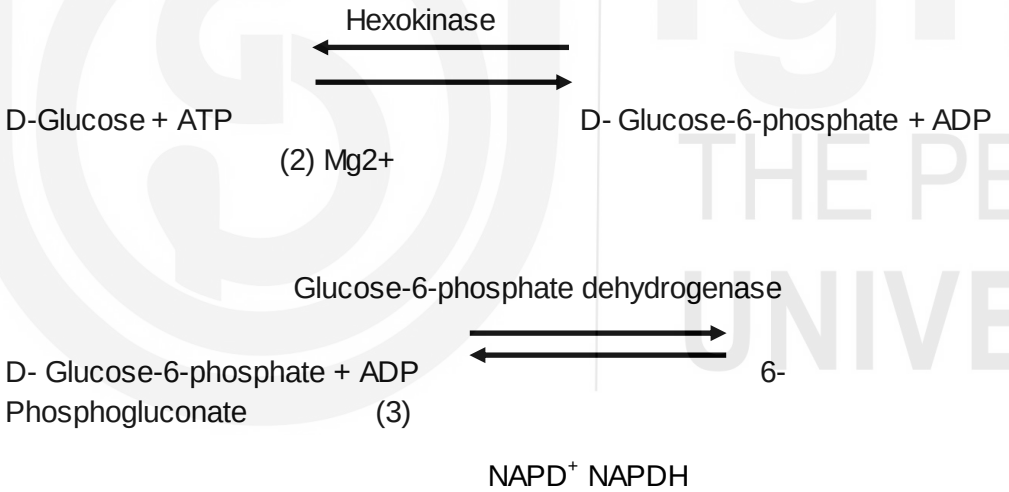


Table 11.1 Diagnostic enzymes and their potential uses.

Enzyme	**Abbreviations**	**Use**
Serum glutamic oxaloacetic Transaminases/ Aspartate transaminase	SGOT/AST	Heart Attack, Liver dysfunction
Serum glutamate-pyruvate Transaminases/ Alanine transaminase	SGPT/ALT	Liver dysfunction

Creatine Kinase	CK	Brain and muscle disorders
Urease		Renal function test
Cholesterol Oxidase		Hypercholesterolemia, Heart attack
Lactate Dehydrogenase	LDH	Myocardial Infraction
Alkaline Phosphatase	ALP	Bile and Liver diseases
Acid Phosphatase	ACP	Prostate Cancer
Glucose Oxidase		Diabetes Mellitus, Hyperglycemia

2. **Immunodiagnostics**: Enzyme immunoassay depends on both immunological and enzymatic reactions, and is classified as heterogenous or homogenous assays.

   - *Heterogeneous assays* use solid-phase support for immobilizing antigens or antibodies, requiring a step to separate analyte and reaction products before analysis using enzymes like peroxidase, β-galactosidase and alkaline phosphatase.

   - *Homogeneous assays:* In these type of assays, enzyme label activity is inhibited either directly through site-specific attachment of a hapten-antibody complex or indirectly by substrates, inhibitors, or cofactors. The free analyte competes for the bound antibody, releasing the inhibited enzyme reaction and generating a signal proportional to the analyte's concentration in the sample. This method simplifies the assay process, generating detection signals directly without separation steps, and offers rapidity, automation, along with minimal separation and washing steps.

3. ***DNA-diagnostics*:** This method involves the multiplication of target molecules and is currently used for detecting nucleic acids like polymerase chain reaction and nucleic acid sequence-base amplification (NASBA). DNA diagnostics are rapidly developing, particularly for identifying genetic, malignant, and infectious diseases, as well as individuals using DNA finger-printing.

11.2.1 Enzymes as Analytical Agents

Enzymes are essential analytical reagents due to their specific nature and efficiency, which enable quantitative determination of substrate concentration. Enzyme metabolism is crucial for all species' survival, and abnormalities can lead to metabolic disorders. Enzymes' biocatalytic properties make them useful in diagnosing various diseases, as they are associated with specific changes in enzyme activity. Elevated enzyme levels in the blood can indicate

liver damage, heart attacks, or muscle disorders (Table 11.2). Enzymes are also preferred markers for detecting diseases like myocardial infarction, jaundice, pancreatitis, cancer, and neurodegenerative disorders, providing insight into the disease process and response therapy assessment. Enzymes are also components of the analytical agents that are used in the diagnosis of certain conditions. Examples of such enzymes are given in the Table 11.2.

Table 11.2: Examples of various enzymes being used as analytical agents

Enzyme	**Diagnosis**
Acid phosphatase	Prostrate carcinoma
Arginase	L- arginine levels in plasma and urine
ALP	Brain neoplasms
Alcohol dehydrogenase	Brain neoplasms
ALT	Hepatitis
Amylase, Cholinesterase lipase (LPS)	Pancreatic disease
Cholesterol esterase/oxidase	Hyper/hypolipedemia
Creatine kinase	Cardiac and skeletal malfunction
Glucose oxidase	Hyper/ hypoglycaemia
Glycerol-3- phosphate dehydrogenase	Triglycerols
Hexokinase/ Glucose-6- phosphate dehydrogenase	Hyper/ hypoglycaemia
Lactate dehydrogenase	Heart attack or liver disease
Lipoprotein lipase	Hyperlipedemia
Urease	Renal function assessment
Glycerol kinase	Hyperlipedemia
Uricase	Gout

SAQ 1

Do as Directed

a) Which of the following enzymes is involved in the diagnosis of gout?

   i) Amylase

   ii) Uricase

   iii) Alcohol dehydrogenase

   iv) Urease

b) Enzymes used for diagnostic purpose involve

   i) Enzyme diagnostics

   ii) Immunodiagnostics

   iii) DNA diagnostics

   iv) All of the above

c) ……. enzyme is used for the diagnosis of heart attack or liver disease (Fill in the Blanks).

d) Identification of genetic diseases involve ……. (Fill in the Blanks).

11.3 APPLICATIONS OF ENZYMES IN MEDICINE

Enzymes play a crucial role in medicine, assisting in diagnosis and treatment. Estimation or determination of the enzyme activities in serum is essential for differential diagnosis, disease prognosis, and early disease detection. As already discussed, enzymes are also vital in extracellular body fluids like blood plasma, urine, digestive juices, amniotic fluid, and cerebrospinal fluid. Enzymes leak through membranes due to energy imbalances, raising fluid levels in the circulatory system. Evaluation and assessment of these enzymes can help to identify damaged cells as well as to indicate the level and extent of injury. Table 11.3 enlists some of these enzymes available that can be used for several types of treatment of diseases:

Table 11.3: Examples of enzymes used in the treatment of diseases

Enzymes	**Treatment**
Algucerase	Gauche's disease Type I
Amylase	Chronic pancreatitis and gastro intestinal disorders
Asparaginase	Acute childhood leukaemia

Enzymes	Treatment
Bacterial asparaginase	Leukaemia
Collagenase	Clean up wounds by removing dead tissues of severe burns and dermal ulcers
Chymotrypsin	Opthamalogy by dissolving the ligaments of the lens at the time of extraction
DNase	Cystic fibrosis
Elastase	Chronic pancreatitis and gastro intestinal disorders
Glutaminase	Leukaemia
Hyaluronidase	Promoting subcutaneously injected drug absorption
α-Lactamase β-Lactamase	Penicillin allergy
Lipase	Chronic pancreatitis and gastro intestinal disorders
Lysozyme	Antibiotic
Penicillinase	Penicillin allergy
Pepsin	Chronic pancreatitis and gastro intestinal disorders
Prolactazyme	Lactose intolerance
Ribonuclease	Antiviral therapy
Streptokinase	Myocardial infarction which dissolves purulent material or the clot
Trypsin peptidase	Chronic pancreatitis and gastro intestinal disorders
Uricase	Gout
Urokinase	Myocardial infarction which dissolves the clot

Therapeutic enzymes have a wide variety of specific uses:

- Oncolytics: Pegasparaginase (Oncaspar) is a medication used to treat patients hypersensitive to L-asparaginase, a substance that affects malignant cells that rely on asparagine for survival. It exploits a metabolic defect in the asparagine synthesis of some malignant cells.
- Diphtheria toxin, an oncolytic enzyme, aids in protein synthesis by transferring NAD's ADP-ribose moiety to elongation factor 2, making it 100–10,000 times more sensitive in tumor cells compared to normal cells. Neuroaminidase removes sialic acid residues from the surface of neoplastic cells, thereby altering their immunogenicity and rendering them sensitive to immune responses.
- Thrombolytics (thrombolytic therapy): The enzymes in this category are streptokinase, tissue plasminogen activator (rt-PA), urokinase, retavase, tenecteplase, and TNK-tPA (TNKaseTM).
- Debridging agents effectively clean open wounds by removing foreign matter and any surrounding dead tissue.
- Proteolytic enzymes also help to degrade host during invasion such as:

  Trypsin (from mammalian pancreas) hydrolyzes peptide bonds involving arginine and lysine;

  Papain (from the leaves and the unripe fruit of the papaya tree) hydrolyzes peptide bonds involving the basic amino acids - lysine, arginine, and histidine.

  Colllagenase (from culture extracts of various animal cells or from various Clostridium species (pathogenic) breakdown collagen at its peptide bond regions under physiological pH and temperature conditions and
- Antiinflammatory agents: Chymotrypsin (a zymogen produced in the pancreas) is converted to active form in the small intestine and Bromelians, which are plant proteases from pineapple stem or fruit, have anti-inflammatory properties due to their ability to break down protein-based inflammatory mediators.

Enzyme replacement therapy provides relief from the following disorders as listed below by supplying missing or deficient enzymes in the body especially in persons with an inherited enzyme deficiency syndrome.

- Metabolic storage disorders due to insufficient activity of housekeeping enzymes.
- Gaucher's disease due to glucocerebrosidase absence (isolated from human placenta) leads to glycolipid accumulation in cells, especially in macrophages.
- Fabry's disease in which kidney, heart, gastrointestinal tract and peripheral nerves are damaged. Fabrazyme restores levels of alpha-galactosidase A to provide relief from the disease.

- Patients suffering from pompe's disease in which the heart, skeletal muscles and brain are involved can seek relief by regular, lifelong infusions of alglucosidase alfa in the form of Lumizyme drug.
- Hurler's disease and Maroteaux- Lamy syndrome in which the eyes, liver, joints and skeleton are usually affected can be treated by Laronidase (Aldurazyme).
    - Digestive aids: Most digestive aid preparations are based on depolymerises responsible from the breakdown of polysaccharides, proteins and lipids. Such preparations may include a single enzyme or multiple enzymes. Some of the examples are given below:
- Alpha amylase hydrolyzes α-1,4-glycosidic bonds, and its oral administration is used to aid digestion.
- Lactase hydrolyzes lactose;
- Various proteolytic enzymes e.g., papain, pepsin;
- Pancreatin (extracted from the pancreas), are used in deficiencies related to the secretion of pancreatic enzymes (e.g., chronic pancreatitis, pancreatic carcinoma, cystic fibrosis).

SAQ 2

Do as Directed

a) Trypsin and papain fall in which of the following categories of enzymes?

i) Anti inflammatory agents

ii) Proteolytic enzymes

iii) Oncolytic enzymes

b) Enzyme replacement therapy is available for which of the following disorders?

i) Gaucher's disease

ii) Niemann Pick disease

iii) Mucolipidosis

iv) Metachromic leukodystrophy

c) …… enzyme is used in antiviral therapy (Fill in the Blanks).

d) …… is an example of anti inflammatory agent (Fill in the Blanks).

11.4 APPLICATIONS OF ENZYMES IN AGRICULTURE

Enzymes are crucial for growth and development in living organisms, including plants, and are increasingly important in agriculture. They play a role in organic matter transformation, nutrient cycle, and uptake, increasing crop production, soil fertility, and food protection. Agricultural enzymes are bioactive proteins that maintain soil health by balancing biological, chemical, and physical components. They break down plant residues and organic matter, providing nutrients and aiding in seed development. The vital role of enzymes in soil can help to downplay ecosystem disturbances thereby aiding sustainable soil management. Soil enzymes are used as indicators of soil health, fertility, and productivity, and can improve crop resistance to water stress and nutrient assimilation. They also act as natural herbicides, pesticides, and fertilizers to enhance crop growth and yield. Enzymes are also sprayed on animal farms to reduce infection and keep insects away.

The most important enzymes used in plant growth and soil fertility includes

Phosphatases, which are crucial in the phosphorus cycle, aiding in plant growth by hydrolyzing phosphates and making phosphorus available in the soil.

Dehydrogenases are responsible for the biological oxidation of soil by hydrolyzing organic material and transferring hydrogen to inorganic acceptors.

Urease breaks down urea-based fertilizers in the soil, providing nitrogen components for plants and absorbing them for soil mineralization. It also decomposes ammonia, nitrite, and nitrate excreted by aquaculture animals, preventing toxic nitrogen waste accumulation in the pond water.

Amylase is a vital enzyme that breaks down starch-based organic substances from food waste, animal waste, and livestock products, improving the environment and habitat of domestic animals, and aiding in their nutritional metabolism, digestion, and growth.

Cellulase breaks down cellulose in plant cell walls into monosaccharides or polysaccharides. Most animals have limited fibre digestion, except herbivores and a few species like termites. Cellulase helps to hydrolyse fibre and carbohydrates in feed ingredients for aquatic animals, promoting nutrient absorption, health, and faster growth. It also treats plant-based organic substances, such as dead algae and wastewater from livestock and aquaculture activities. Only a few species produce cellulase to consume fibre.

Researchers have identified enzymes involved in producing auxin, a plant growth hormone that impacts cell division and flowering.

In agronomy, soil microbes and their enzymes are crucial for assessing soil health, fertility, and crop yield. They play a role in nitrogen, sulphur, and phosphorus cycling, as well as organic waste degradation. Enzyme-microbe complexes are sustainable ways to increase crop production and soil fertility, leading to improvement in the crop quality.

SAQ 3

Do as Directed

a) Which of the following enzymes is involved in providing nitrogen requirement to plants?

   i) Amylase

   ii) Urease

   iii) Cellulase

   iv) Phosphatase

b) Cellulase breaks down cellulose in plant cellwall to

   i) Monosaccharides

   ii) Disaccharides

   iii) Oligosaccharides

c) Phosphatase help in hydrolysing……..in the soil (Fill in the Blanks).

d) Microbes are preferred sources of enzymes than plants and animals (True/False).

11.5 APPLICATIONS OF ENZYMES IN RESEARCH

11.5.1 Immobilized Enzymes

Nowadays immobilized enzymes are being used increasingly in the industry and research due to their ability to be used continuously and reused without a difficult recovery process. You will read more in detail about the different processes of enzyme immobilization in the last unit of this course**.** Some of the examples of well-known immobilized enzymes and their applications are given in the Table 11.4

Table 11.4: Immobilized enzymes and their applications

Immobilized Enzyme	**Application**
Amino acid acylase	Resolution of racemic mixture
Aspartase L-Arginine deaminase Histidine ammonia lyase	Aminoacid interconversion

Immobilized Enzyme	Application
α-Amylase	Production of corn syrup
β-Lactase	Production of lactose- free milk
Exo-1,4-α-D-glucosidase Xylose isomerase	Production of High -fructose corn syrup from starch
Flavoprotein oxidase	Nitrogen oxidation of drugs containing hydrazine groups
Flavoprotein oxidase	Production of prednisolone
Glucose coamylase	Production of glucose from corn syrup
Glucose oxidase	Fabrication of Biosensors e.g., Glucose biosensor
Invertase	Production of invert sugar
Invertase Inulinase	Conversion of sucrose into glucose and fructose
L-amino acid oxidase	D-amino acid production
Lipase	Production of cocoa butter substitute
Nitrile hydratase	Production of Acrylamide
Pectinate	Treatment of fruit juice
Penicillin amidase	Production of precursors of semi-synthetic penicillin
Protease	Hydrolysis of whey proteins, cheese ripening
Ribonuclease	Nucleotide production from RNA
Tannase	Hydrolysis of tannins

Immobilized Enzyme	Application
Thermolysin	Production of Artificial Sweetening Agent
Urease	Estimation of urea

11.5.2 Biocatalytic Applications

Immobilized enzymes from microorganisms have high catalytic efficiencies with a wide range of extensive applications (Table 11.5).

Table 11.5: Immobilized enzymes as biocatalytic agents

Immobilized Enzyme	Applications
Alcohol dehydrogenase	Biotransformation
Acetylation	Biotransformation in the production of antibiotics
Cytochrome P450 (Drug Metabolising Enzymes)	Biotransformation of drugs in the body
Hydroxylation	Biotransformation of steroids

11.6 DIAGNOSTIC ENZYMES

Diagnostic enzymes are enzymes used for disease detection, diagnosis, or prognosis. They are used as reagents to determine normal and pathological components in substances like serum, urine, and gastric juice. These enzymes are often used in assays and tests to diagnose diseases, monitor health conditions, or evaluate the effectiveness of treatments. The enzymes are divided into three groups:

-*Cellular enzymes:* These enzymes enter blood from various organs, and their activity in serum depends on their content, molecular weight, intracellular localization, and rate of elimination.

-*Secretory enzymes*: These enzymes are synthesized by cells and then enter the blood stream, and fulfill their specific functions in the circulatory system. These are the enzymes of the coagulation system and fibrinolysis, choline esterase, etc.

-*Excretory enzymes*: These enzymes are synthesized by the glands of the GIT and enter the blood (e.g., amylase, lipase).

Enzymes, located in various cellular compartments, can indicate the severity of cellular damage. Diseased states often cause moderate or extensive tissue damage. The bloodstream releases enzymes specific to diseased organs, exhibiting enhanced activity. Measurement of enzyme activities in blood, plasma, or other body fluids is used for diagnosis, as it has better sensitivity and accuracy, and changes in enzyme activity can be detected earlier than clinical signs or other diagnostic indicators.

Commonly used enzymes in clinical practice aid in diagnosing, prognosing, and monitoring pathologies, with their determination in blood serum having high clinical significance. Some of the well-known examples of diagnostic enzymes are listed below:

11.6.1 Amino Transferases (Transaminases)

These are a group of enzymes that carry out the interconversion of amino acids and oxyacids by transferring amino groups. Two types of amino transferases are used in diagnostic enzymology. These are alanine amino transferase (ALT), formally called glutamate pyruvate amino transferase (GPT), and aspartate amino transferase (AST), formerly called glutamate oxaloacetate amino transferase (GOT).

Alanine amino transferase (ALT): ALT speeds up reversible transamination of L-amino acid and 2-oxoglutamate into pyruvate and glutamate inside the cell's cytoplasm. ALT is a key indicator of liver, heart, and muscle damage. It's a key marker in diagnosing liver disorders like hepatitis, hepatocirrhosis, viral hepatitis, and toxic liver necrosis. Elevated ALT levels are linked to an increased risk of hepatocellular carcinoma. In healthy adults, ALT concentrations range from 5–35 U/L, while higher levels indicate diseased organs.

Aspartate amino transferase (AST): AST is an enzyme that converts L-aspartate and 2-oxoglutarate into oxaloacetate and glutamate. It is found in various tissues and can be used to monitor heart and muscle diseases. The AST-to-platelet ratio index can also be used to screen for liver fibrosis in chronic hepatitis B. Elevated AST levels are more specific than ALT in evaluating hepatic disorders in large animals, but ALT plasma levels may exceed those of AST in hepatitis.

11.6.2 Alkaline Phosphatase (ALP)

ALP is an enzyme that breaks down phosphate ester bonds from nucleotides and proteins under alkaline conditions, leading to its increased serum levels. It is primarily produced in the liver, bones, intestine, and kidney. The placenta in pregnant women produces ALP. Serum ALP levels are higher in children and correlate with bone growth rates. Men have slightly higher ALP values than women, but after age 60, they are equal to or higher than women. ALP concentrations increase during puberty, pregnancy, and after menopause. Serum ALP activity is increased in bone and liver diseases, including osteomalacia, rickets, Paget's disease, carcinoma, cholestasis, lesions, tumours, hyperthyroidism, and hyperparathyroidism. Higher levels of ALP are also linked to hepatic fibrosis in patients with NASH and type 2 diabetes. Low

ALP activity is less common and more likely to be related to genetic conditions or nutritional deficiency.

11.6.3 Acid Phosphatase (ACP)

Acid phosphatase, a protein found in the body, is primarily found in the prostate gland, where it is 100 times more abundant than in other tissues. It is used to monitor prostatic cancer progress. There are five types of ACP, including prostatic, erythrocytic, macrophagic, lysosomal, and osteoclastic. ACP is more concentrated in semen, making it a common test for rape victims. Increased ACP activity is also observed in patients with Gaucher's disease.

11.6.4 Lactate Dehydrogenase (LDH)

This enzyme is present in all body cells but is most abundant in the liver, heart, pancreas, and kidney, catalyzing the reversible interconversion of pyruvate and lactate. There are five well defined LDH isoenzymes according to the type of tissues in which they reside:

LDH1 in the heart and red blood cells;

LDH2 in white blood cells;

`LDH3 in the lungs;

LDH4 in the kidney; and

LDH5 in the liver and skeletal muscles.

Serum electrophoresis is the primary method for identifying isoenzymes, which are tetramers of H and M, which can form different isoenzymes. Elevations of LDH1 and LDH2 are common after myocardial infarction, acute leukaemia, and liver or skeletal muscle damage.

LDH gene expression is unregulated in various human malignant tumours, including colorectal, lung, breast, oral, prostate, germ cell, and pancreatic cancers. It serves as a prognostic marker for acute leukaemia and sickle cell disease, and is crucial for testicular germ cell malignancy monitoring.

11.6.5 Creatine Kinase (CK)

Creatine kinase (CK) is an intracellular enzyme that converts creatine phosphate to ADP to generate ATP after muscle cell depletion. It consists of three isoenzymes: CK-MM, CK-MB, and CK-BB. CK-MM is found in skeletal muscles and increases in cases of heart, brain, and skeletal damage. CK-MB is present in the heart and increases after heart attacks, inflammation, and muscle dystrophy. CK-BB is mostly found in the brain and increases in cases of brain injury, meningitis, abnormal cell growth, stroke, and hyperthermia. CK levels can increase to up to 2.0 U/ml during myocardial infarction, muscular dystrophy, and inflammatory reactions.

11.6.6 Amylase

Salivary alpha (α) amylase is a digestive enzyme found in high concentrations in pancreatic juice and saliva. It cleaves starch and glycogen into maltose,

making it a sensitive marker in acute pancreatitis. Additionally, it serves as a biomarker for heart failure, chronic stress, and kidney function monitoring. Some important enzymes of clinical significance are listed in the Table 11.6:

Table 11.6: Distribution and application of clinically important enzymes

Enzymes	Tissues	Clinical applications
5'- nucleotidase	Liver	Pancreatic diseases
Alanine amino transferase	Liver	Hepatoparenchymal diseases
Alkaline phosphatase	Liver, bone, intestinal mucosa, placenta	Liver and bone diseases
Amylase	Salivary glands, pancreas	Pancreatic diseases
Aspartate amino transferase	Liver, skeletal muscle, heart, erythrocytes	Hepatic parenchymal disease, muscle disease
Cholinesterase	Liver	Organophosphorus insecticide poisoning
Creatine kinase	Skeletal muscle, heart	Muscle disease
Gamma glutamyl transferase	Liver	Hepatobillary disease, Marker alcohol abuse
Lipase	Pancreas	Pancreatic diseases
LDH	Heart, liver, skeletal muscle, erythrocytes, lymph nodes, platelets	Hepatoparenchymal diseases, muscle diseases, hemolysis, tumour markers

SAQ 1

Do as Directed

a) Which of the following enzyme is not used to diagnose hepatobilliary disease?

i) Alkaline phosphatase

ii) 5'-nucleotidase

iii) Alanine aminotransferase

iv) α-glutamyl transferase

b) The group of enzymes that catalyze the interconversion of amino acids to keto acids by transfer of amino groups is referred to as

i) Alkaline phosphatase

ii) 5'-nucleotidase

iii) Transaminase

iv) α-glutamyl transferase

c) ……. hydrolyses α-1,4-linkages in starch and glycogen to yield maltose (Fill in the Blanks).

d) An increase in serum level of LDH1 relative to LDH2 is an indication of …….. (Fill in the Blanks).

11.7 ENZYME IMMUNOASSAY

How diagnostic enzymes are used in clinical laboratories? What do you think? The answer is that several labs run ELISA assays using diagnostic enzymes to determine or to monitor the presence of specific antigens or antibodies in a given sample. Enzyme immunoassay methods are sensitive and versatile, serving as an alternative to radioisotopes in immunoassays. They can determine protein and hormone levels, and are not hazardous to health. Enzymes can be detected using more accessible techniques. Two common examples of enzyme immunoassay (EIA) procedures are:

-Enzyme- linked immunosorbent assay (ELISA)

- Enzyme multiplied immunoassay test (EMIT)

11.7.1 ELISA

ELISA is a solid-phase enzyme immunoassay used to detect the presence of a specific protein in blood samples. It uses enzymes like ACP, Horse Radish Peroxidase (HRP), lactoperoxidase, and α-galactosidase to detect the binding of an antigen or antibody.

- *Antigen-antibody reactions,* in which the presence of antigen or antibody is detected in a sample;

  Enzymatic chemical reactions determine antigen or antibody quantity by determining the antigen-antibody complex formation rate catalyzing a colorless substrate to produce a colored product. and

- *Signal detection and quantification*, in which the intensity of the colored product generated by the enzyme and substrate is detected and measured.

ELISA serves as a diagnostic tool for diagnosing both non-infectious diseases such as hormones, drugs, and autoimmune ones, and infectious diseases

such as HIV, Lyme disease, tuberculosis, Rocky Mountain spotted fever, rotavirus, squamous cell carcinoma, syphilis, and toxoplasmosis.

11.7.2 EMIT

The enzyme multiplied immunoassay technique (EMIT) is a method used for determining therapeutic and recreational drugs and proteins in serum and urine. It involves a drug competing with an enzyme to bind to an antibody, with an increased drug concentration causing a color change.

Immunoassays are used in life science research to track proteins, hormones, and antibodies, and in industry to detect contaminants in food and water. They differ from enzyme-linked immunosorbent assays (ELISA) in two ways: enzymes play a key role throughout the assay process and require little enzyme technology knowledge. The EMIT therapeutic drug monitoring tests provide accurate information on drug concentrations, such as immunosuppressant drugs and antibiotics.

SAQ 5

Do as Directed

a) Which of the following techniques are used in enzyme immunoassay?

   i) Immunofluorescence

   ii) Radio immunoassay

   iii) ELISA

b) In an enzyme immunoassay, the enzyme

   i) is bound by the antibody's antigen-binding site

   ii) is attached to the wall of micro titre plate

   iii) is conjugated to the suspect antigen

   iv) is bound to the constant region of the secondary antibody

c) ELISA allows for rapid screening and quantification of the presence of…… in the sample (Fill in the Blanks).

d) ELISA stands for …….(Fill in the Blanks).

11.8 ENZYME THERAPY

Enzyme therapy is the use of enzymes to treat medical conditions such as enzyme deficiencies and other health issues. In the enzyme therapy, enzymes are added to change the existing abnormal or diseased state owing to the lack or short supply of same enzyme in the body. Enzymes are given most likely in an immobilized manner but biodegradable form. Enzymes aid in digestion, detoxification, strengthens immune system, muscle contraction, and reduces stress on vital organs like the pancreas. It has numerous applications,

including treatment of pancreatic insufficiency, cystic fibrosis, metabolic disorders, lactose intolerance, and cancers. Enzyme therapy has been involved in the degradation of necrotic tissue by usage of proteolytic enzymes such as trypsin or chymotrypsin. Enzymes can be administered orally, topically, or intravenously and can be non-systematic or systematic.

Enzymes have also been used as drugs in the treatment of specific medical problems.

- **Streptokinase,** an enzyme from Streptococcus, is crucial for clearing blood clots in the lower extremities and treating heart attacks by activating the fibrinolytic proenzyme plasminogen, which attacks fibrin and breaks it down into soluble components.
- **Asparaginase therapy** is used for certain adult leukaemia types, as tumour cells require asparagine, which is depressed by administering asparaginase, thereby affecting their viability.
- **Micro-plasminogen activator**, formerly urokinase, is an enzyme extracted from human urine that can be infused into the bloodstream of individuals at risk for pulmonary embolism. It stimulates a cascade system for active plasmin, a proteolytic enzyme that digests fibrin.
- **Immobilized enzymes:** Artificial kidney machines use immobilized enzymes to remove waste products from the body, such as urea, due to kidney disease. Urea enters the machine through harm-dialysis, converted to CO_2 and NH_4^+ by immobilized urease, and toxic NH_4^+ is trapped on resins or incorporated into glutamate for coenzyme recycling before returning to the bloodstream.
- Enzyme therapy has been used to diagnose of necrotic tissue using proteolytic enzymes such as **trypsin** or **chymotrypsin**.
- **Adenosine deaminase (ADA)** is utilized in treating inherited immunological disorders by breaking down excess adenosine in patients' blood, thereby reducing the damage to the immune system.
- **α-glucocerebrosidase**, an enzyme involved in glycolipid metabolism, causes Gaucher's disease, a lysosomal storage disorder characterized by glucocerebroside accumulation due to gene mutations.
- **Sacrosidase** treats congenital sucrose-isomaltase deficiency (CSID), enabling patients to digest sucrose, a disaccharide, and consume sucrose-containing foods, thereby improving their digestive abilities.
- Enzymes are used as **digestive aids,** i.e., to cure the digestive problems induced by some sugars.

- The α-galactosidase enzyme is used as a digestive aid for individuals experiencing bloating, gas, and diarrhea after consuming foods like beans and Brassica vegetables. It breaks down the terminal α-galactosidic residue of sugar substrates, potentially causing discomfort due to bacterial fermentation.

- Lactose supplements can treat lactose intolerance, a condition where insufficient lactase enzyme breaks down milk sugar lactose into glucose and galactose.

- PKU is an inheritable disorder caused by the absence of the phenyl alanine hydroxylase enzyme, which converts phenyl alanine to tyrosine. The recombinant yeast phenyl alanine ammonia lyase (PAL) is an oral treatment for this disorder.

- Lysozyme, a naturally produced bactericidal enzyme, has been found to selectively degrade viral RNA, making it a promising candidate for HIV treatment.

Limitations

The bulky structure and large molecular weight of enzymes exclude them from intracellular domains, and their high antigenicity causes rapid clearance from blood plasma. Parenteral enzymes require extensive purification which leads to higher costs.

Enzymes and Inborn errors of Metabolism: Inborn errors of metabolism occur due to genetic mutations and are characterized by the loss of enzyme activity. More than 400 errors are known but they occur rarely, for example phenylketonuria, 1 in 12000 births; cystinuria, cystinuria, 1 in 14,000; galactosemia, 1 in 57,000. These errors are identified by enzyme assays or the presence or absence of particular metabolite in blood, tissue, urine or other biological samples. The advent of recombinant technology has led to the development of highly accurate and sensitive diagnostic procedure for determining enzyme activities. It is feasible to obtain a pre-natal diagnosis by carrying out enzyme assays in the amniotic fluid. Some examples (Table 11.7) of inborn errors of metabolism due to enzyme deficiencies are as given below:

Table 11.7: Enzyme deficiency and inborn errors of metabolism

Inborn Error	Enzyme Deficiency
Alkaptonuria	Homogentisate1,2 –dioxygenase
Phenylketonuria	Phenylalanine 4 monooxygenase
Galactosemia	Galactose 1-phosphate uridyltransferase
Glycogen storage disease type V	Glycogen phosphorylase
Tay-Sachs	β-N-Acetyl-D-hexosaminidase

Treatment of inborn errors of metabolism usually involves reduction in the intake of substances that cannot be metabolized normally or supplementation of special diets to the patients. However, this kind of treatment is ineffective because of endogenous synthesis of metabolites in question or due to blockage of particular metabolic pathway vital for the body function. A successful treatment is possible only when normal enzyme could be administered at the appropriate site for example urokinase plasminogen activator (uPA) extracted from human urine can be infused in the patients suffering from pulmonary embolism. L-Asparaginase is also used in the treatment of leukemia.

SAQ 6

Do as Directed

a) Which of the following enzymes are not used in the treatment of gastrointestinal tract disorder and chronic pancreatitis?

   i) Pepsin

   ii) Cellulase

   iii) Amylase

   iv) Trypsin

b) Bacterial asparaginase is used in the treatment of

   i) Cleaning of wounds

   ii) Venus thrombosis

   iii) Leukemia

   iv) Traumatic or post operative edema

c) To dissolve blood clots during myocardial infarction, ……. enzyme is used (Fill in the Blanks).

d) Another name of urokinase is …….. (Fill in the Blanks).

11.9 Taq POLYMERASE

Taq polymerase itself isn't generally used directly as a diagnostic enzyme in the similar manner as enzymes like lactate dehydrogenase or creatine kinase, it plays a vital role in several diagnostic applications through its usage in PCR-based methods.

11.9.1 Characterization of Taq DNA Polymerase

Taq DNA polymerase is a heat-stable enzyme from Thermus aquaticus, with the highest activity at a pH of 9 and 75ºC. It incorporates nucleotides at 2-4 kilobases per minute and was discovered in Yellowstone National Park in 1969. Despite lacking 5' to 3' and 3' to 5' exonuclease activities, it is ideal for

manual and automated DNA sequencing due to its fast, progressive nature, and broad temperature range.

- *Size, activity, and fidelity*: Taq, a DNA polymerase, has a C-terminus activity and 5' to 3' exonuclease activity, with an approximate size of 94 kDa.
- *Temperature dependency*: Taq's optimal temperature range is 75-80ºC, where its catalytic activity can reach 150 nucleosides per second due to its temperature-dependence.
- *Thermostability*: Taq, a heat-stable PCR tool, can be used in hot-start protocols, but its half-life decreases rapidly at high temperatures, causing PCR protocols with denaturing steps at 95ºC to denature a portion of the Taq.
- *Monovalent and divalent cation dependencies*: Monovalent and divalent ions, like K^+ and Mg^{2+}, neutralise DNA's negative charges, reducing repulsive interactions. They are added in salt forms like KCl and $MgCl_2$, respectively. Divalent cations, like Mg^{2+} or Mn^{2+}, act as cofactors during polymerization, with $MgCl_2$ concentrations around 2 mM.
- *pH dependency*: The enzyme's optimal pH range is 8-9.4 units, varying with the buffer system. Common commercially available Taq buffers typically contain 10-50mM Tris-HCl for pH stability.

11.9.2 Applications

Taq polymerase is generally not considered a diagnostic enzyme per se but being integrated to PCR based diagnostic methods, it becomes essential for several range of molecular diagnostic applications. Some of these molecular applications are:

1. **PCR Amplification:**
    - **Disease Detection:** In clinical laboratories, during PCR, Taq polymerase is used to amplify DNA sequences from several pathogens (bacteria, viruses, etc.) in samples. For example, PCR can identify the presence of viral DNA in infections such as HIV, hepatitis, or SARS-CoV-2 (the virus causing COVID-19).
    - **Genetic Testing:** PCR can amplify specific genes or genetic mutations. It will be extremely helpful in diagnosing genetic disorders, determining hereditary conditions, thereby guiding personalized medicine.
2. **Quantitative PCR (qPCR):**
    - **Quantification of Nucleic Acids:** qPCR as the name suggests permits quantitative measurement of DNA or RNA in a sample. It uses Taq polymerase to determine and assess the viral load in infections, measurement of gene expression levels, or determination of the concentration of a specific genetic marker.

3. **Molecular Diagnostics:**

   - **Pathogen Identification:** PCR assays using Taq polymerase can identify definite pathogens by amplifying their exclusive genetic material. This application helps diagnose infectious diseases quickly and accurately.
   - **Biomarker Detection:** PCR-based techniques can detect biomarkers related to diseases, such as cancer-related gene mutations or specific bacterial or viral DNA.

11.9.3 Limitations

- Taq's performance is limited in challenging applications like high-fidelity synthesis of long amplicons and amplification of GC-rich sequences.
- Taq DNA polymerase's low disincorporation rate, estimated between one and 10,000 bases, is due to its lack of 3'-5' proof-reading activity.

SAQ 7

Do as Directed

a) Taq polymerase is a ——- polymerase

   i) Heat stable

   ii) Buffering

   iii) Denaturant

   iv) Large

b) …….. is used to extract the polymerase for PCR (Fill in the Blanks).

c) qPCR as the name suggests permits quantitative measurement of …….. in a sample (Fill in the Blanks).

11.10 ENZYME ELECTRODES AND ENZYME AS BIOSENSORS

Biosensors, or enzyme electrodes, are electronic devices that sense and analyse biological information using enzyme specificity and immobilization techniques. Biosensors have found an extensive usage in the analytical chemistry. They are used by autoanalysers to estimate biochemical and pathological quantities in biological samples like plasma, serum, urine, and cerebrospinal fluid by converting the biological response to an electronic signal which is amplified, processed and displayed. Biosensor technology uses enzyme specificity and reactivity to transform metabolites into products, determining substrate usage or product composition. Biosensors offer reliability, sensitivity, accuracy, ease of handling, and low cost compared to conventional detection methods, making them ideal for biomedical

applications like medical diagnostics, pathogen detection, food safety control, and environmental monitoring.

11.10.1 Components of a Biosensor

A biosensor is an analytical device that uses a biological or biologically derived sensing material to detect an analyte. It consists of a *biorecognition* device and a *biotransducer*, which converts biochemical activity into electrical energy (Fig. 11.1). The analyte diffuses into the enzyme layer, where catalytic reactions occur. The signal is processed by a *processor* and displayed on a screen, allowing for a better understanding of the analyte's presence and concentration.

The immobilization method, including adsorption, cross-linking, and self-assembly, significantly impacts enzyme activity and biosensor sensitivity, with enzymes incorporated into carbon-paste, polymers, and gels.

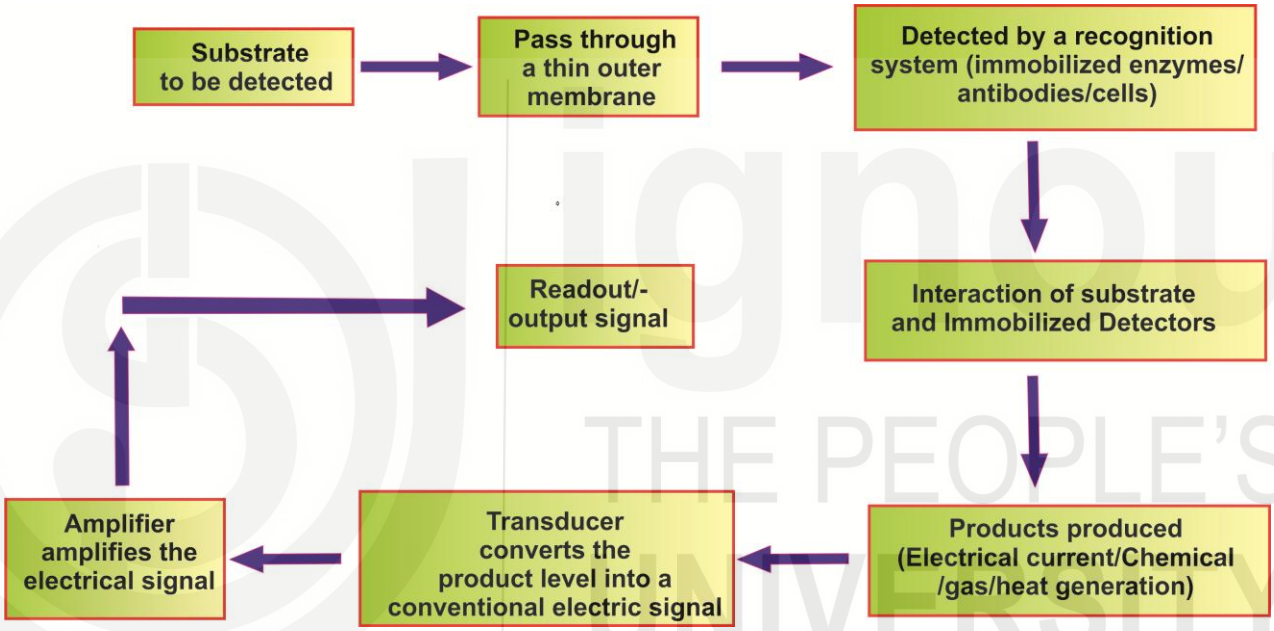


Fig. 11.1: Components of Biosensor.

Enzyme-based biosensors have been developed over time, using various transducer systems and various shapes and sizes, as shown in Table 11.8.

Table 11.8: Types of Biosensors

Transducer	Example
Calometric	Thermistor, Thermopile
Electrochemical	Clark electrode, Mediated electrode, Ion selective electrode
Magnetic	Bead-based devices
Optical	Photodiode, Waveguide system, Integrated optical devices

Transducer	Example
Piezoelectric	Quartz crystal microbalance (QCM), Surface acoustic wave (SAW) devices

11.10.2 Types of Biosensors

There are different types of biosensors having different applications:

- *Calorimetric biosensor*: Calorimetric biosensors measure temperature changes in turbid and strongly colored solutions but maintain a constant sample temperature, making them widely applicable.
- *Potentiometric biosensor*: The altered electron distribution at the transducer surface produces an electrical potential, converting the biological reaction into an electric signal.
- *Amperometric biosensors*: Amperometric biosensors detect electron movement through redox reactions, producing current proportional to substrate concentration. They reduce reagent costs and enhance measuring devices' amplification.
- *Optical biosensor*: Optical biosensors measure light produced or absorbed during a reaction by analysing the change in fluorescence or absorbance caused by catalytic reactions.
- *Acoustic biosensors*: Piezoelectric devices, also known as transducers, detect changes in the mass of biological components due to reactions. Because they are covered in antibodies, they bind to complementary antigens in sample solutions. This lowers the vibrational frequency and lets you know how much of an antigen is present.

11.10.3 Examples of Biosensors

Some of the widely used biosensors are discussed below:

Biosensors for Blood Glucose:

The biosensor is a valuable tool for glucose sensor research, measuring blood glucose concentration, a crucial aspect for diabetes monitoring due to limited enzymes.

- Glucose dehydrogenase-based glucose biosensors,
- Cellulose dehydrogenase-based glucose biosensors,
- Glucose-oxidase-based glucose biosensors.

Diabetic patients must monitor their blood glucose levels to detect potential hyperglycemia and hypoglycemia. Glucose measurements involve the interaction of enzymes like hexokinase, glucose oxidase (GOD), and glucose-1-dehydrogenase (GDH). GOD (immobilized on a polyacrylamide gel), used in biosensors, catalyzes the oxidation of α-D-glucose to gluconic acid using molecular oxygen with simultaneous production of H_2O_2.

$$\text{Glucose} + O_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{Catalase}} 1/2\ O_2 + H_2O$$

Glucose concentration can be measured through O_2 consumption or H_2O_2 production, and temperature rise due to energy released in reactions.

Biosensors for the measurement of urea in blood or urine: Researchers have developed sensors based on urease enzyme to accurately measure urea in urine or blood samples for diagnosing renal and liver disorders, as urease converts urea to hydrogen carbonate and ammonium ions.

$$(NH_2)_2CO + 2H_2O + H^+ \rightarrow 2NH_4^+ + HCO_3^-$$

The urease biosensor is created by immobilizing urease in a bovine serum albumin membrane on the surface of an ion-sensitive field-effect transistor (ISFET).

Biosensors for lactic acid: Lactate sensors, based on lactic acid dehydrogenase, detect oxygen deficits in muscles and liver, indicating tissue oxygenation and warning of ischemic conditions. Surgical procedures, intensive therapy, sports medicine, and spatial medicine utilize these biosensors. Different biosensors use immobilized lactate monooxygenase (LMO) and lactate oxidase (LOD) for monitoring lactate levels. Two other types of lactate biosensors are

- *Ex vivo biosensors,* equipped with microdialysis or ultrafiltration probes that measure blood lactic acid by diffusing it into the dialysate/filtrate for external measurement.
- *In vivo biosensors* measure blood lactate concentration directly, responding quickly to lactate level changes, either through skin placement or subcutaneous implantation.
- The rapid response to lactate concentration changes is comparable to that of ex vivo biosensors, but biocompatibility requirements are more stringent.

Biosensor for Creatinine: These are based on creatine amidinohydrolase, sarcosine oxidase, and creatine amidohydrolase. Creatinine estimation is crucial for diagnosing renal, thyroid, muscle function, acute myocardial infarction, and hemodialysis therapy. Creatinine biosensors use creatinine deaminase, which catalyzes a reaction, making them essential for diagnosing acute myocardial infarction and describing hemodialysis therapy. Creatinine biosensors generally employ creatinine deaminase, which catalyses the following reaction.

$$\text{Creatinine} + H_2O \longrightarrow \text{N-Methylhyadantoin} + NH_4^+$$

Allosteric enzyme based biosensor: Allosteric enzymes regulate catalytic activities by binding effector molecules to different receptor sites, forming easily detectable products and serving as biosensors.

Modular engineering can integrate allosteric enzymes into biosensors, using their receptor site as the recognition element and their active site as the transducer. However, most of their modulators are not analytically relevant, making them unsuitable for direct use. Some allosteric enzyme-based biosensors are listed below:

- α-galactosidase
- Alkaline phosphatase
- L-Lactamase
- Neutral protease
- Ribozymes

A summary of some recent applications of enzyme-based sensors in clinical diagnosis is presented in the table below (Table 11.9).

Table 11.9: Examples of enzyme- based biosensors

Sensor	**Immobilized enzyme**
Acetyl choline and Choline	Acetyl choline esterase, Choline oxidase
Amino acid	Amino acid oxidase
Arginine	Arginase
Asparagine	Asparaginase
Bilirubin	Hemoglobin, Glucose oxidase
Carnitine	Carnitine dehydrogenase and diaphorase
Cholesterol	Cholesterol oxidase
Creatine and Creatinine	Creatinase, Creatininase, Sarcosine oxidase
Ethanol	Alcohol Dehydrogenase
Glucose	Glucose oxidase/Glucose dehydrogenase
Glutamate	Glutamate oxidase/Glutaminase

Sensor	Immobilized enzyme
Lactate	Lactate oxidase/Lactate dehydrogenase
Oxalic acid	Oxalate oxidase
Penicillin	Penicillinase
Theophylline	Theophylline oxidase
Triglycerides	Glycerol kinase and glycerol phosphate oxidase
Urea	Urease
Uric acid	Uricase
γ-Amino butyric acid	Catalase, Glutamate oxidase

11.10.4 Advantages of Enzyme Biosensors

Enzyme biosensors offer a number of advantages:

- Negligible enzyme consumption, which is in the range of a few milliunits per sample;
- Ability to quantify the diagnostically important metabolites without purifying them from other impurities that may otherwise interfere with the diagnosis;
- Advantage of measuring labile metabolites without degradation as it happens with chemical methods;
- Highly specific as compared to cell-based sensors;
- Short reaction time, or faster response.

11.10.5 Disadvantages of Enzyme Biosensors

- The high cost of purified enzymes may prohibit the use of diagnostic methods compared to other diagnostic tools,
- Inactivation of enzymes during the enzymatic process by inhibitors present in the sample or other ingredients.
- They are too expensive to produce as an additional step in isolating the enzyme.
- The stability of enzymes is hindered during isolation.
- Cofactors are required for the detection of substances.

11.10.6 Limitations of Enzyme Electrodes

Enzyme electrodes are simple to operate, allowing for simple sample pre-treatment and analysis, but they have several limitations.

- *Optimal sample frequency*: Enzyme-electrode-based analysis has a maximum sample throughput of 100 samples per hour, with response based on cell compartment concentration-time profile and internal diffusion time at high loading.
- *Extension of new substances*: The number of substances directly determined with a monoenzyme electrode is limited due to electrochemical inactivity in many enzyme-catalyzed reactions, necessitating readily measurable substances to be formed sequentially or parallel to analyte conversion.
- *Adapted sensitivity*: The sensitivity of an enzyme electrode can be improved by substrate amplification, which involves adjusting operational conditions to enable one enzyme to regenerate the substrate of the other, using oxidase and dehydrogenase couplings.

SAQ 8

a) Which of the following feature is not processed by biosensor?

   i) The biocatalyst used in the biosensor must be highly specific for the purpose of the analyses.

   ii) The reaction occurring in the biosensor should be as independent of such physical parameters.

   iii) Active site is mainly constituted by non-polar amino acids for catalysis to take place.

   iv) The response from the biosensors should be accurate, precise, reproducible and linear.

b) Immobilized enzymes are more preferred over free enzymes in producing biosensors.

   i) True

   ii) False

c) When the physical change produces in a biosensor is due to the movement of electrons produced in the redox reaction, the biosensor is referred to as …… (Fill in the Blanks).

d) In the biosensor, …….. processor is one which involves subtracting a ‘reference’ baseline from the sample signal (Fill in the Blanks).

11.11 SUMMARY

Let us summarize:

- Enzymology includes the diagnostics of enzymes as a subfield. The methodology encompasses two primary objectives: firstly, the utilization of enzymes as reagents to identify normal and pathological components in biological samples (e.g., gastric juice, urine, and serum), thereby functioning as indicators of cellular damage; and secondly, enzymatic activity measurement in biological material.
- These days there is an increasing trend towards the use of enzymes in clinical diagnosis, and in enzyme therapy. These trends are due to increased availability of the sophisticated automated equipment in clinical laboratories.
- A wide variety of purified enzymes are becoming commercially available in larger quantities due to recombinant DNA technology using bacterial and other expression systems, and many of these have been successfully immobilized to support materials. Due to these reasons, assay of metabolites and enzyme replacement therapy is becoming more economical.
- Immobilized enzymes are being used increasingly in the industry and research due to their ability to be used continuously and reused without a difficult recovery process.
- Diagnostic enzymes are enzymes used for disease detection, diagnosis, or prognosis. They are used as reagents to determine normal and pathological components in substances like serum, urine, and gastric juice. These enzymes are often used in assays and tests to diagnose diseases, monitor health conditions, or evaluate the effectiveness of treatments.
- Agricultural enzymes are bioactive proteins that maintain soil health by balancing biological, chemical, and physical components. They break down plant residues and organic matter, providing nutrients and aiding in seed development.
- Enzyme immunoassay methods are sensitive, versatile, and are used to determine protein and hormone levels. Two common examples of enzyme immunoassay (EIA) procedures are

Enzyme- linked immunosorbent assay (ELISA) and Enzyme multiplied immunoassay test (EMIT).

- Enzyme therapy is the use of enzymes to treat medical conditions such as enzyme deficiencies and other health issues. In the enzyme therapy, enzymes are added to change the existing abnormal or diseased state owing to the lack or short supply of same enzyme in the body.
- Biosensors, or enzyme electrodes, are electronic devices that sense and analyse biological information using enzyme specificity and immobilization techniques. Biosensors have found an extensive usage in the analytical chemistry. They are used by autoanalysers to estimate biochemical and pathological quantities in biological samples like plasma, serum, urine, and cerebrospinal fluid by converting the

biological response to an electronic signal which is amplified, processed and displayed.

11.12 TERMINAL QUESTIONS

1. How enzymes help in the diagnosis of a disease? Give examples.
2. Discuss the therapeutic applications of enzymes.
3. How do enzymes help in the growth and development of plants?
4. What are diagnostic enzymes? Discuss with examples.
5. Write a note on ELISA.
6. What is meant by enzyme therapy? Give examples.
7. Discuss the characteristics of Taq polymerase.
8. Explain the components of a biosensor.

11.13 ANSWERS

1. a) Option (ii), b) Option (iv), c) Lactate dehydrogenase, d) DNA diagnostics
2. a) Option (ii), b) Option (i), c) Ribonuclease, d) Chymotrypsin
3. a) Option (ii), b) Option (i), c) phosphates, d) Option (i)
4. a) Option (iii), b) Option (iii), c) Alpha amylase, d) myocardial infarction
5. a) Option (iii), b) Option (iv), c) antigen, d) Enzyme-linked immunosorbent assay
6. a) Option (iv), b) Option (iii), c) streptokinase, d) Micro-plasminogen activator
7. a) Option (i), b) Thermus aquaticus, c) DNA or RNA
8. a) Option (iii), b) Option (i), c) amperometric biosensor, d) signal

Terminal Answers

1. Discussed in Section 11.2

2. Enzymes have been widely utilized in the therapeutic industry of the present day and are essential for the detection, prevention, and biochemical analysis of severe diseases. The treatment facility has been significantly enhanced through the use of enzymes, which also aid in the acceleration of biochemical reactions within living organisms and the improvement of the human body's emulative system. Examples are discussed in Section 11.3.

3. Refer to Section 11.4.

4. Refer to Section 11.6.

5. Refer to Section 11.7.1.
6. Discussed in Section 11.8.
7. Discussed in Section 11.9.1.
8. Discussed in Section 11.10.1.

11.14 FURTHER READINGS

1. Prasad NK. Enzyme Technology. Pacemaker of Biotechnology. 2011. PHI Learning Pvt. Ltd.
2. Singh S. A Textbook of Enzymes. 2007. Campus Books.
3. Grewal S, Mutha P. Enzyme Technology. 2010. Agrobios (Indoa).
4. Palmer T. Enzymes: Biochemistry, Biotechnology and Clinical Chemistry. 2004. East-West Press Put. Ltd, New Delhi.
5. Chauhan BS. Comprehensive Enzyme Technology. 2005. Satya Prakashan, New Delhi.
6. Price NC, Stevens L. Fundamentals of Enzymology. The Cell and Molecular Biology of Catalytic Proteins. 2010. 3rd Edition. Oxford University Press.
7. Dubey RC. Advanced Biotechnology. 2014. S Hand & Company Pvt. Ltd.
8. Pathak A. Tools and Techniques in Radiation Biophysics. 2023. Springer Publisher.

UNIT 12

APPLICATION OF ENZYMES

Structure

12.1 INTRODUCTION

In the previous unit, you have come across the remarkable applications of enzymes in diagnostics. What makes enzymes so attractive for industry? As you know, enzymes speed up the rate of reactions, production processes could be performed at a fraction of time at much lower temperature and pressure. These advantages along with several others such as specific catalytic activity, undesirable side reactions and their operability under mild conditions make the enzymes highly desirable in comparison to other chemical catalysts. Moreover, the usage of enzymes is not new. Role of several enzymes especially in starch, cheese making, brewing and textile industries has been carried out centuries before their biochemical aspect was understood. Microorganisms are natural and most preferred sources for industrial enzymes due to easy availability, and fast growth rate. With the advent of genetic engineering there has been a revolution in the field of enzyme technology.

The enzymes present in number of bacteria and fungi were found for many years by man but it was only in late 1970s isolation of enzymes was carried out and their commercial aspects began to be explored. The separation, isolation and purification of enzymes along with their immobilization have

opened opportunities and challenges to scientists in the field of enzyme technology as well as in application of enzymes. Now-a-days a wide range of enzymes are available on a much bigger scale and a much lesser cost. As the whole area of enzymes is expanding, the industrial and commercial application of enzyme is also increasing. The estimated market of enzymes in 2018 is approximately 4500 million dollars.

The present unit will give you an overview on several applications of enzyme in different processes and industries.

Expected Learning Outcomes

After studying this unit, you should be able to:

- describe the usage of enzymes in detergent industry;
- illustrate the role of enzymes in food industry such as starch;
- state the role of enzymes in baking industry;
- explain the commercial viability of enzymes in wine and brewing industries; and
- enumerate several other applications of enzymes and their potential commercial uses.

12.2 APPLICATION OF ENZYMES

As you know applications of enzymes are of immense value. An estimation of world enzyme market in 2018 put the figure roughly in approximately 4500 million dollars. The effective catalytic properties of enzymes have already promoted their introduction in several industrial products and processes. Genetic manipulation, site directed mutagenesis, protein engineering, cloning and genetic recombination along with several other techniques in biotechnology have proved extremely useful to develop new version of enzymes with better stability and catalytic efficiency. Enzymes are now tailor made to suit the needs of medical and technical industry with vast range of applications. Several enzymes are extensively used in textile, pulp, detergent, beer, bakery and starch industries. Enzymes also have tremendous therapeutic and diagnostic value. Now-a-days large scale production of enzymes is also needed for research purposes.

Microbial sources are the ideal choice for protein isolation and purification. Microorganisms are preferred over animal or plant as sources for enzyme production. They can be easily grown in large numbers in bioreactors at nominal cost using simple nutrients. They can also withstand extreme pH, temperature or dissolved oxygen like conditions. Enzymes, particularly from microbial sources can be cultured largely by gene manipulations, as per the need for industrial applications. Applications of microbial enzymes in food, pharmaceutical, textile, paper, leather, and other industries are numerous and increasing rapidly over conventional methods due to several advantages such

as less harm to the environment, greater efficiency, and the higher quality products.

Microbial strains that produces enzymes certain enzymes are the ideal choice or target for commercial viability. The microorganisms can be easily screened and selected for producing the desired enzyme. The procedure is economical too. Furthermore, it would be advantageous if the organism does not produce any toxic compounds. Higher and better yield of enzyme without any production of unwanted toxic molecule make the particular strain of microorganisms a better choice than other sources of enzyme. Isolation and purification of a thermostable enzyme is more convenient than other thermosensitive enzymes. The thermophilic bacteria and fungi are the ideal sources of enzymes for industrial or commercial use.

Other sources of enzymes are plant and animal tissues. Plants are excellent sources of enzymes such as papain. Animal tissues contain several enzymes such as lipases, esterases and proteases. However, there are several limitations in isolating enzymes from plant and animal sources. Non-microbial sources of enzymes plant and animal tissues have damaging materials such as phenolic compounds and endogenous enzyme inhibitors and proteases. The quantities of enzyme in plant and animal tissues are limited and highly varied in their distribution. With the advent of plant and animal tissue culture nowadays the possibility of producing large quantities of enzymes is being explored. The constraining factor is their high cost. Several proteins such as vaccines are being produced in large numbers using tissue culture techniques. Presently 80 % of the commercial enzymes are from microbial sources. The following table (Table 12.1) shows the sources of enzymes along with their relative contribution in the commercial enzymes.

Table 12.1: Source of enzymes and their contribution in commercial enzymes.

Source of enzyme	Contribution (in %)
Fungi	60
Bacteria	24
Yeast	04
Streptomyces	02
Higher Animals	06
Higher Plants	04

Though several enzymes are used in the industry using whole microbial organism as a source of enzymes yet, the efficiency and efficacy of industrial production processes can be enhanced using isolated enzymes. Extracellular enzymes are a better choice than the intracellular enzymes because of their ease of isolation and purification. Do you know why? The isolation and purification of extracellular enzymes is much simpler and cheaper. The useful enzymes are separated, isolated and purified to increase the catalytic activity

and better yield of the product. The inherent disadvantages of these processes also involve cost in separation and isolation as well as purification processes of enzyme or inactivation of the enzyme.

The methods available for isolation of proteins or enzymes are generally the same. You have come across these techniques for isolation and purification of enzymes in the previous unit Unit-10 of this course.

SAQ 1

Answer the following:

a) Why microbes are considered as better sources of enzymes?

b) Name two sources of enzymes other than microorganisms.

c) Which source of enzyme will be better- extracellular enzyme or intracellular enzyme?

12.2.1 Enzymes in Detergent Industry

Detergent industry is one of the largest single markets for enzymes. Alkaline proteases, amylases and lipases from microbial sources are being used because of their stability at high temperature, alkaline pH and reduction of agitation period. The dirt on clothes includes many compounds including proteins, lipids and starch. Blood stains as well as stains contaminated with milk or oils can be effectively and efficiently removed from the clothes by detergent enzymes. Utilization of detergents in water at high temperatures with rigorous mixing makes it possible to remove most of dirt. The microbial enzymes are proving to be cost effective and also lead to less energy consumption. Home detergents include both an amylase and a protease. Enzymes in granulated forms as well as liquid preparations are used in very small amounts in the detergent industry. Some of the home detergents include amylase as well as protease enzyme as these can be further used for laundering lot of dirty clothes and fabrics. Industrial laundering is large and requires high level of cleanliness with minimal usage of water. Newer versions of detergents include alkaline and thermostable enzymes as fungal cellulase which generally removes small fibers thereby restoring tensile strength of the fabric. Bacterial proteases from Bacillus licheniformis, fungal amylases and lipases from Aspergillus niger, Rhizopus niveus are widely used in the detergent industries.

12.2.2 Enzymes in Starch Industry

Starch industry developed rapidly after 1990s. Starch containing crops such as maize, potato and wheat are a vital ingredient of food consumed by the world's population. Starch containing parts of the plant needs to be processed to harvest the starch. Starch is used in different food products as glucose, maltose, high fructose corn syrups and starch hydrolysates. Plant products such as maize, potato and wheat are well known sources of starch for commercial purposes. Several enzymes involved in processing of starch are

endoamylases, exoamylases, debranching enzymes and transferases. A pictorial representation of the enzymes (Fig.12.1) involved in starch processing and its several products is as given below:

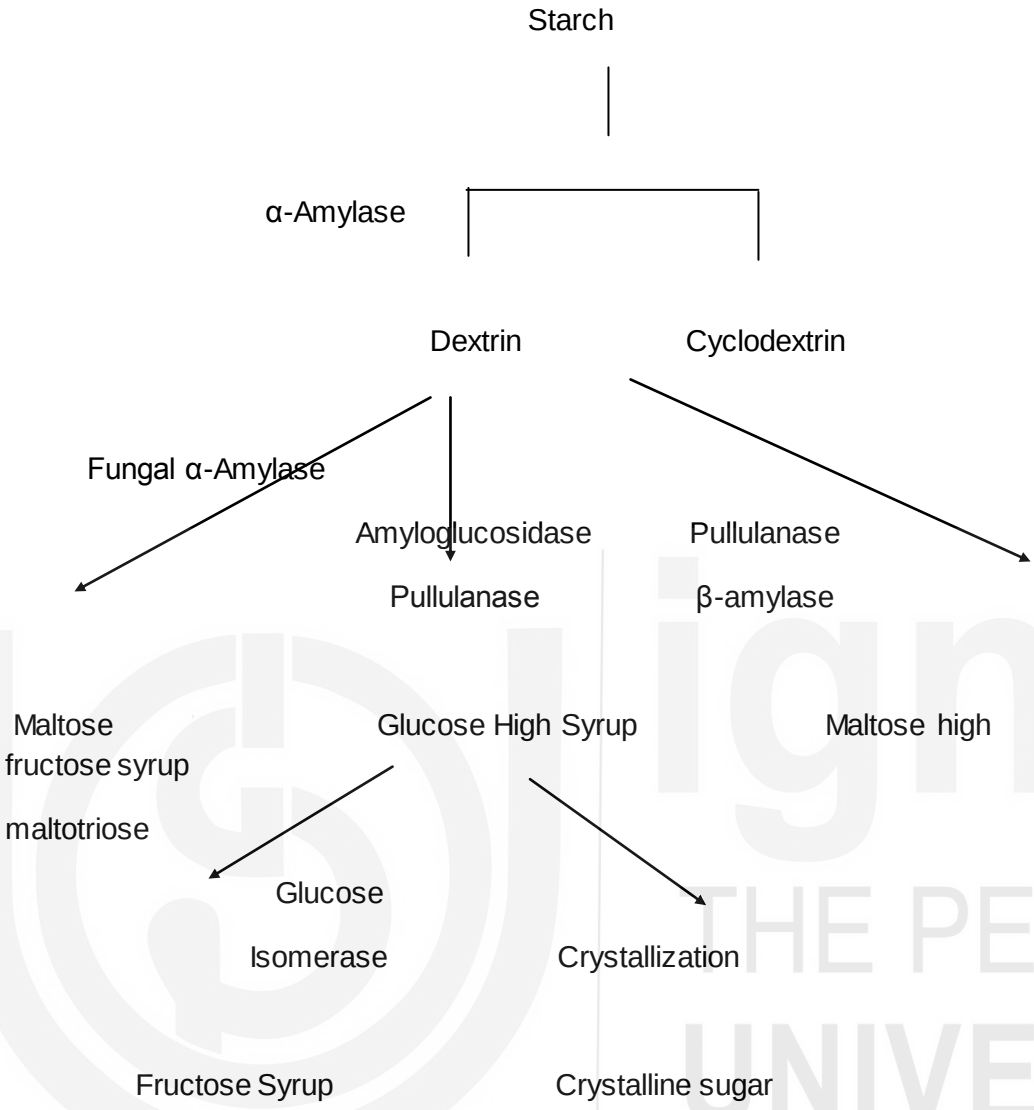


Fig. 12.1: Enzymes of starch industry.

In the unit of carbohydrates, you might have come across starch, a polymer of glucose units which are linked via α-glycosidic linkage involving C1 and C4 hydroxyl groups. Generally, starch is present in the form of granules. These granules differ widely in size and physical characteristics. Endoamylases cleave α-(1→4) glycosidic bonds present in amylase or amylopectin chain in starch. They are found in bacteria and Archaea. Similarly, exoamylases cleave exclusively α-(1→4) and α-(1→6) glycosidic bonds. They act on external glucose residues of amylase or amylopectin to produce glucose. Starch converting enzymes such as isoamylases and pullulanases constitute another group of starch processing enzymes. They are mainly debranching enzymes that cleave the-(1→6) glycosidic bonds. Transferases are also starch converting enzymes that cleave α-(1→4) glycosidic bonds and transfer a part of the donor to a glycosidic acceptor along with formation of new glycosidic bond.

The production of glucose syrup from starch begins by dissolving starch granules in the water by boiling or using jet cooker. The heated starch slurry is passed into a hydrolysis reactor for enzymatic treatment. The degree of starch sweetness depends on the degree of hydrolysis. Generally, the production of maltose or glucose syrup from starch has three stages: gelatinization, liquefaction and saccharification. In industries, high glucose syrup can be converted to high fructose syrup using enzyme D-xylose-ketol isomerase. Fructose, as you know is an isomer of glucose. It is twice as sweet as glucose.

12.2.3 Enzymes in Baking Industry

Bread baking industries are now seeking an extensive usage of enzymes. Starch modifying enzymes are playing a key role in baking and its different type of applications. Flour components are hydrolyzed by different enzymes for example amylase hydrolyses starch, xylanases degrades xylan, proteases degrade proteins and lipases hydrolyse the lipids. Oxidative enzymes like glucose oxidase and lipoxygenase also act on different components of flour. These enzymes affect the doughness or other properties of bread. Xylanase increases the loaf volume and along with amylase gives softer morsel and extended shelf life. Cell wall hydrolyzing enzymes such as cellulase, pectinase and xylanase also play an important role in baking. Xylanases and glucanses improve the elasticity of rye bread. Hemicellulases makes the cell wall more soluble in wheat dough. Wheat flour includes about 1-1.5 % lipids. Non-polar lipids such as triglycerides are bound with gluten. The addition of fungal lipase hydrolyses the triglycerides which are bound to gluten and modify their interactions in wheat flour. It leads to the formation of stable dough and improved morsel structure. Fungal amylase derived from Aspergillus oryzae in dough increases the loaf volume and improved texture of the baked product. Glucose oxidase from *Aspergilus niger* and *Penicillium* are known to oxidize alpha-D-glucose to gluconon-1,5-lactone. It helps in improving the storage capability of flour.

Enzymes are used in the making of biscuits and nuts. The soft flour used in making biscuits is different from those in bread making. It is basically a form of dough with more elasticity in which gluten should not be too strong. Generally, sodium bisulphate is used to produce the desired gluten but it is becoming unpopular because of its potential health hazards. Sodium bisulphite has been banned in many countries. Instead thermo stable proteases from Bacillus subtilis are used nowadays to solve this problem by softening the gluten without affecting the quality of dough.

SAQ 2

Answer the following:

a) Isomerase is used to change glucose syrup into fructose syrup (True/False).

b) Amylase and protease enzymes are used for laundering lot of dirty clothes and fabrics (True/False).

c) Proteases degrade lipids in the baking industry (True/False).

d) The soft flour used in making biscuits is not different from those in bread making. (True/False).

12.2.4 Enzymes in Fruit juice, Brewing and Wine Industry

Do you take fruit juices? Do you anticipate the role of enzymes in fruit juices? Fruit juice industry is rapidly increasing nowadays. The industry faces several problems in maintaining the stability of fruit juices. The fruit juices turn turbid or cloudy due to the presence of compounds like cellulose, hemicelluloses, xylans and arabans. The problem is even more acute with citrus fruits. Pectic compounds are complex colloidal acid polysaccharides constituting α-(1-->4) galacturonic acid residues with side chains consisting of xylose, galactose or L-rhamnose or arabinose. When the tissue is grinded, pectin rich fruit gives fruit juice with high viscosity. The usage of pectinases helps to lessen the viscosity of fruit juice and increases the pressability of pulp. The yield of fruit juice also increases significantly. Apple juice is filtered clear by the usage of pectic enzymes. They help in processing of the fruit pulp as well as in fruit juice extraction. Pectinase such as rhamnogalacturonase aids in the maceration of apple tissue. Pectinases depolymerizes highly esterified pectin. Combination of pectinesterases, polygalactouronases, polymethylegalactouronate lyases improves the clarification of pear juice. Orange juice includes large amounts of pectin esterase to take away methoxyl groups from pectin molecules. Mango fruit is extensively used for making juice or squash. Mango nectar has 25-30 % squash of pH 3.5 and titrable acidity of 0.2-0.4 %. Pectinases, hemicellulases and cellulases enzymes assist in degrading mango pulp to make a plain, non-gelatinous and clarified juice. Similarly, a mixture of enzymes like pectinesterase, arabanase, hemicellulase, tannase and cellulose are used to clarify the guava juice. Some of the bitter compounds present in citrus fruits especially grapes are naringin, limonin and neohesperidin. The enzyme naringinase from several species of microorganisms catalyzes the hydrolysis of naringin to rhamnose and naringenin (a non-bitter derivative). Naringinase is an enzyme complex constituting L-rhamnosidase and glucosidase and therefore helps to remove rhamnose and glucose from naringin.

Beer is made by mixing barley malt with hot water in large vessel using mashing. Maize, sorghum, rice and barley and other starchy cereals are also added to the mash. The filtered mash is boiled and cooled and then transferred to fermentation vessels and thereafter yeast is added in these vessels. After fermentation, green beer is matured for additional filtration and bottling. The enzymes used to convert cereals to beer are barley malt. This malted barley is a major source of enzymes in the brewing process of wine industries. Acidic pectinases from fungal sources such as Aspergillus niger are widely used in fruit juice and wine industries. Under optimal conditions, activation of many enzymes such as α-amylases, carboxypeptidases and β-glucanase leads to hydrolysis of starch materials in the seed reserve which is eventually used for the beer production. Bacterial and fungal amylase enzymes are added for saccharification. Pectinases and hemicellulases

enzymes play a significant role in the wine industry as they help in clarification, filtration and also improve wine quality and stability.

12.2.5 Enzymes in Food Industry

Proteases enzyme are being used in food industry from number of years. Papain from the leaves of unripe fruit helps in tenderizing the meat. Rennet from the stomach of calves is traditionally used for cheese production. Proteases aids in extraction of proteins from the dead animals for the preparation of canned meats and soups. You must have heard of soya sauce used extensively in fast food industry. Proteolysis in soya protein can increase the value of their usage in the making of soya sauce. A partial proteolysis augments whipping expansion of soya protein and its emulsifying properties.

Proteases help to recover proteins from animal tissues after butchering. The bones from the animal are mashed with alkaline proteases and incubated at 65^{o}C up to four hours. The meat slurry formed is used in canned meats and soups.

The animal feed industry is a significant division of agro-business worth more than 100 billion dollars. The major components of this industry include poultry, pigs and ruminants, pet foods and fish farming. Majority of the food constituents are not entirely digested by livestock. The application of enzymes increases their digestibility. The enzymes can be added to the feed directly or premixed with vitamins, minerals or other food additives. Hydrolases eliminates the anti-nutritional factors present in the grains or vegetables. These enzymes are known to increase the digestibility of animal feed as well as for enhancing the nutritional value of feed. They also supplement animals with their own digestive enzymes like amylases and β-glucanases to hydrolyse non-starchy polysaccharides such as barley. The non-starchy polysaccharides in cereals results in poor feed conversion rate, slow weight gain and sticky droppings by young chicks. Enzymes such as β-glucanases and xylanases degrade these polysaccharides improving their digestion as well as absorption to increase weight gain and egg laying hens.

Cellulases and hemicellulases have great potential applications in the animal feed industry. The increase milk yield and body weight by ruminants. These enzymes hydrolyze plant cell wall polysaccharides since the fodder diet of ruminants contains cellulose, hemicelluloses, pectin and lignin. Dairy cows have shown a 5-20 % increase in the production of milk when they are fed with forage treated with fibrinolytic enzymes.

12.2.6 Enzyme in Other Industries

Textile, paper and pulp industry uses chemical reagents of diverse composition. Its dyestuff such as benzidine and other aromatic compounds are known to be carcinogenic. The dye wastewater is treated with ligninolytic enzymes like lignin peroxidase and manganese peroxidase to degrade dyes, including synthetic ones used in the industry. Thermostable enzymes like cellulases, xylanases and proteases are also used in producing good quality yarn or cotton. The material used in making fabrics contains cellulosic fibres such as cotton, linen, viscose and lyocell. These materials from 'fuzz (short

fibers sticking out from the surface)' or become fluffy. These shortcomings in the cellulosic fibres can be eliminated using cellulases in process called as bio-polishing. This process is carried out during textile wet processing stage that includes desizing, bleaching, dyeing and finishing. The enzyme cellulases act on small fibres and polish them. They also help in smoothening, increasing the moisture absorbance and giving glossy appearance to the fabrics. Similarly, acid cellulases have demonstrated to be successful in treating lyocell garments by giving them a soft feel look and preventing piling. Fungal α-amylases are also used in desizing woven fabrics without harming the yarn.

Enzymes are used to enhance deinking and bleach in paper and pulp industry. Paper industry involves separation and degradation of lignin in the wood pulp. The new environment issues stress the need to replace conventional pollution emanating chlorine-based delignification/bleaching processes. The treatment of wood pulp with ligninolytic enzymes addresses this apprehension by providing gentle and cleaner strategies of delignification. The usage of enzymes in bio bleaching systems is also providing additional help to the paper industry. Another category of enzymes like lacasses can be used in targeted modification of wood fibres. Enzymes such as cellulases and hemicellulases are also used in paper and pulp industry for several purposes. Cellulases are used for the development of the bioprocess for recycling of used printed papers. Mechanical pulping in refining and grinding of the woody raw material provide pulps with elevated content of fines, bulk and stiffness but the processes involve high-energy consumption. Bio-mechanical pulping results in substantial energy savings. In bio-bleaching processes, pretreatment of lignocellulosic materials with microorganisms seems promising because microorganisms produce hemicellulolytic enzymes (xylanases) that limits the hydrolysis of hemicelluloses in pulp to increase the extractability of lignin.

The milk and dairy products are one of the oldest industries which remain applicable till today. The world's adult population is around 75 % today. The probable market of mild and dairy products industry is still being recognized. Fluid milk, cheese products, milk powder, condensed milk and ice cream are various products of dairy food industry. The use of enzymes (proteases, lipases, esterases, lactase, aminopeptidase, lysozyme, lactoperoxidase, transglutaminase, catalase, etc.) in dairy market is well acknowledged and varies from coagulant to bio-protective enzyme to enhance the shelf life and safety of dairy products. The commercial production and development of enzyme galactosidase (lactase) from microbial sources has made dairy industry significantly practicable. If you recall from your unit on carbohydrates, lactose is a disaccharide made of D-glucose and D-galactose. Lactase from microorganisms hydrolyzes lactose. It can be produced commercially from dairy yeast *Kluyeromyces fragilis* at pH optimum (6.5-7.2) with milk treatment. Yeast β-galactosidases are used for lactose hydrolysis in milk and sweet whey. Milk acidification is required for release of whey from the cheese. Lactic acid bacteria such as *Lactobacillus sp* convert lactose to lactic acid. Several varieties of cheese can be made using starter 'a culture inoculum containing lactic acid bacteria'. Casein fraction of milk can be coagulated to form a gel by lowering the milk pH and adding up rennet 'mixture containing specific proteolytic enzymes'. Chymosin enzyme can coagulate about 70 % of the

cheese. Several species of lactobacillus such as l. bulgaricus, L. lactis, L. casei, L. Plantarum and Streptococcus such as S. cremoris, S. thermophilus are commonlu used for the cheese production.

Discharges and waste disposed from different stages of leather processing in leather industry are causing severe health hazards and environmental problems. The biodegradable enzymes are effective substitutes to improve the quality of leather and help to shrink waste. The largest process in leather preparation of dehairing require large amount of enzymes like proteases, lipases and amylases. Alkaline proteases such as subtilisin from Bacillus subtilis with pH optima of 9-10 are commonly used to clean the stock and to facilitate uptake of water uptake of the hide or skin. Enzyme proteases cause breakdown of fibrous proteins like proteoglycans, dermatan sulphate and keratin sulfate present in the extracellular matrix (ECM) as well as smoothens the progress of removal of salts and hyaluronic acid. Lipases help in dispersion of fats and production of waterproof and low fogging leathers. After dehairing, hides to be used for soft leather clothing are bated using specific enzymes such as elastase, keratinase and collegenase present in ECM. Bacillus licheniformis and B. amyloliquefaciens are extensively used to produce proteases. Enzymes are also required for facilitation and enhancement of leather quality during different stages in leather processing, such as curing, soaking, liming, dehairing, bating, picking, degreasing and tanning.

Extensive application of enzymes has not left the cosmetic industry untouched. The applications of enzymes in cosmetics have been increasing continuously. Anti oxidative enzymes are well known free radical scavengers and have therefore find their extensive applications in sunscreen cream, toothpaste, mouthwashes, hair waving and dyeing. Superoxide dismutase (SOD) is used to apprehend free radicals and to control skin damage caused by pollutants present in air and water, microbes and other harmful factors. SOD and peroxidases are used in combination in sunscreen cream as free radical scavengers to reduce erythema. Proteases in skin creams help to clean and smoothen the skin through peeling off dead or damaged skin.

Toothpaste and mouthwash nowadays contain enzymes such as endoglycosidase and papain to whiten teeth, to remove plaque and to remove odor-causing deposits on teeth and gum tissue. Several enzymes like laccase, oxidases, peroxidases, and polyphenol oxidases are used in hair dyeing. Lipase, catalase, papain, bromelain and subtilisin enzymes have found their usage in skin care. Protein disulfide isomerase, glutathione sulfhydryl oxidase and transglutaminase are used for hair waving. Latest usage of enzymes includes their role as contact lens cleaners to remove protein films.

SAQ 3

Answer the following:

a) Pectinases polymerize/depolymerize highly esterified pectin (choose one option)

b) Name two enzymes used in producing good quality yarn or cotton.

c) …… and ……..enzymes play a significant role in clarification and improvement of wine quality and stability.

d) Hydrolases eliminates the antinutritional/ nutritional factors present in the grains or vegetables (choose one option)

12.2.7 Enzymes in Waste Water Management

With increasing population, domestic waste is ever increasing. The industrial effluents as well as domestic waste have several pollutants that are not only hazardous but toxic to the living beings and ecosystem. Waste water management is important for the ecosystem and a number of enzymes that are found to be involved in the degradation of toxic pollutants are proving their potential as saviors. Enzymes have been found to have enormous potential in waste water treatment by increasing biological oxygen demand (BOD) and chemical oxygen demand (COD).

Laccases, polyphenol oxidases, lignin peroxidases, reductases, methyl transferases and cytochrome oxygenases play a big and vital role in the degradation of toxic xenobiotics. Microbial enzymes alone or in combinations are successfully used for the treatment of industrial effluents containing phenols, aromatic amines, nitriles, etc. Enzymes employed for waste treatment includes amidases, amylases, amyloglucosidases, cellulases, glucoamylases, lipases, nitrile hydratases, pectinases and proteases. Oxidoreductases causes detoxification of toxic organic compounds through oxidative coupling. These enzymes, like laccase, manganese peroxidase, lignin peroxidase and tyrosinase helps to catalyze the elimination of chlorinated phenolic compounds from industrial effluents.

Microbial oxygenases, such as monooxygenases and dioxygenases have an extensive substrate range, and are therefore active against a broad range of compounds, including the chlorinated aliphatics. Degradation of halogenated organic compounds containing pollutants, such as insecticides, fungicides, herbicides, plasticizers, hydraulic fluids as well as several intermediates for chemical synthesis can be carried out using these microbial enzymes.

12.3 SUMMARY

Let us summarize application of enzymes in industry:

- Practical interest in enzyme processes has led to the development of enzyme industry which runs in hundreds of millions. Enzymes are used commercially for the reason that of their catalytic abilities. Enzymes are beneficial because of low costs and their usage in minimal quantities.
- The applications of enzymes are huge and varied for example in food, starch, detergent and baking industry.

- Alkaline proteases, amylases and lipases from microbial sources are extensively used in the detergent industry because of their stability at high temperature, alkaline pH and reduction of agitation period.
- Starch is used in different food products as glucose, maltose, high fructose corn syrups and starch hydrolysates. Several enzymes involved in processing of starch include endoamylases, exoamylases, debranching enzymes and transferases.
- Cell wall hydrolyzing enzymes such as cellulase, pectinase and xylanase play a significant role in the baking industry.
- Enzymes also help in processing of the fruit pulp as well as in fruit juice extraction. Pectinase such as rhamnogalacturonase aids in the maceration of apple tissue. Pectinases depolymerizes highly esterified pectin. Combination of pectinesterases, polygalactouronases, polymethylegalactouronate lyases improves the clarification of pear juice.
- In food industry, enzymes such as amylases, lactases and cellulases break composite sugars to simple ones. Papain from the leaves of unripe fruit helps in tenderizing the meat. Rennet from the stomach of calves is traditionally used for cheese production. Proteases aids in extraction of proteins from the dead animals for the preparation of canned meats and soups.
- Enzymes have enormous potential in various industrial sectors such as leather processing and paper & pulp. They are used to enhance deinking and bleaching in paper and pulp industry. Paper industry involves separation and degradation of lignin in the wood pulp. The new environment issues stress the need to replace conventional pollution emanating chlorine-based delignification/bleaching processes. Enzymes applications in these prospective areas are proving boon to the industrial sector.
- The use of enzymes (proteases, lipases, esterases, lactase, aminopeptidase, lysozyme, lactoperoxidase, transglutaminase, catalase, etc.) in dairy market is well acknowledged and varies from coagulant to bio-protective enzyme to enhance the shelf life and safety of dairy products.
- Anti oxidative enzymes are well known free radical scavengers and have therefore find their extensive applications in sunscreen cream, toothpaste, mouthwashes, hair waving and dyeing.
- Enzymes have significant potential in waste management, and consequently in the development of green environment.

12.5 TERMINAL QUESTIONS

1. What makes enzyme catalyzed reactions more suitable for industrial applications?
2. Describe the role of enzymes in the starch industry.
3. Describe how biological washing powders work.
4. Discuss the disadvantages in the usage of enzymes.

11.6 ANSWERS

Self-Assessment Questions

1. a) Microbes can be easily grown in large numbers in bioreactors at nominal cost using simple nutrients. They can also withstand extreme pH, temperature or dissolved oxygen like conditions. So, these are ideal choice as enzyme source.
   b) Plants, Animals
   c) Extracellular enzyme
2. a) True, b)True, c)False, d) False
3. a) Depolymerizes
   b) Cellulases, xylanases, proteases
   c) Pectinases and hemicellulases
   d) Anti-nutritional

Terminal Questions

1. Industrialists were the first to discover the potential of enzymes as they realized if the reactions are needed to be speeded up production processes could be performed in only a fraction of the normal time or at low temperature or pressure or using economical starting material. As a result, enzymes are finding widespread usage from bio-bleaching of paper to efficient recovery of oil and gas. Usage of enzyme is considered to be safe, cost-effective or environment friendly. Food, detergent or beverage industries are heavily dependent on enzymes as well as the role enzymes in bio-analysis have become extensive nowadays.
2. Refer to section 12.2.2
3. Biological washing powders contain lipases and proteases which breakdown the proteins and fats in stains. For details refer to section 12.2.1
4. Enzymes are highly sensitive to the surrounding environment which makes them hard to use. They get denatured due to small changes in pH and temperature. They are also highly susceptible to poisons. Isolation and purification of enzymes involve huge costs and complex

separation techniques. Enzymes substrate mixture must be uncontaminated as other substances in the reaction mixture may affect the reaction. All these above mentioned disadvantages make usage of enzymes very hard as the conditions involving them must be tightly controlled.

12.7 FURTHER READINGS

1. David L. Nelson and Michael M. Cox: Lehninger Principles of Biochemistry 6th Ed., W.H. Freeman.
2. Robert K. Murray, Daryl K. Granner, Victor W. Rodwell Harper's Illustrated Biochemistry, 27th edition. 2006, McGraw-Hill.
3. Donald J Voet and Judith G. Voet:Principles of Biochemistry4th ed., John Wiley and Sons, Inc, USA.
4. Eric E Conn, Paul K Stumpf: Outlines of Biochemistry, Jophn Wiley and Sons, Inc, USA.
5. S. Shanmugan and T. Sathishkumar: Enzyme Technology, I K International Publishing House Pvt Ltd, New Delhi.
6. Nicholas C Price and Lewis Stevens: Fundamentals of Enzymology, Oxford University Press, Oxford, New York, USA.

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UNIT 13

IMMOBILIZED ENZYMES AND ENZYME ENGINEERING

Structure

13.1 INTRODUCTION

Despite high catalytic efficiency widespread applications of enzymes is hindered by lack of operational stability, short shelf life and cumbersome recovery. All these factors pose major challenge for the industry. Think from a commercial point of view; is usage of enzymes cost effective? The answer would be no unless we have an enzyme technology that involves reuse of enzyme for different commercial purposes. How will it be possible? The answer lies in the enzyme immobilization and enzyme engineering.

An enzyme that is physically attached to an inert solid support is known as immobilized enzyme.

Immobilization is a process in which enzymes are attached to or within solid supports thereby generating a heterogeneous immobilized enzyme system. The solid support helps the enzymes to maintain their activities. Immobilized enzymes imitate their natural mode in living cells, where most of them are attached to cellular cytoskeleton, membrane, and organelle structures.

Immobilization benefits include increased resistance of enzymes to the changes in conditions such as temperature or pH. Recent developments in the field of immobilization technology and its potential commercial applications have renewed interest in million-dollar food and pharmaceutical industries worldwide. This unit discusses the fundamental concept of enzyme

immobilization, methods of enzyme immobilization as well as its several applications.

Enzyme engineering has developed as a useful tool to overcome the limitations of native enzymes as biocatalysts. It is the procedure of enhancing enzyme efficiency or an increased enzyme activity by modifying or changing its amino acid sequence. Several approaches are being utilized for engineering enzymes. Rational design, Semi-rational and directed (molecular) evolution are the three general approaches in enzyme engineering. Lately, genetic engineering techniques are extensively used to improve enzyme efficiency. For exploring enzyme sequences and for creating new and efficient biocatalysts, the combination of directed evolution and rational protein design using computational tools is becoming increasingly relevant.

Expected Learning Outcomes

After studying this unit, you should be able to:

- describe the concept, properties and importance of enzyme immobilization,
- determine different types of immobilization,
- find out how enzyme can be immobilized,
- explain the binding mode of enzyme,
- discuss the potential uses of immobilized enzymes,
- distinguish different methods of enzyme engineering.

13.2 ENZYME IMOBILIZATION-TYPES AND METHODS

Immobilization of enzyme increases its commercial value. These enzymes are more stable and easy to handle. They are also more robust and resistant to environmental changes in comparison to free enzymes. Another advantage of using heterogeneous immobilized enzymes systems is that they allow the effortless recovery of both enzymes and products along with multiple reuses of enzymes. Immobilization ensures nonstop operation of enzymatic processes, speedy termination of reactions and better range of bioreactor designs. These potential uses along with non contamination of reaction products with enzymes make them highly lucrative for industry. In some cases of immobilized enzymes such as proteases reduced levels of autolysis process proves highly beneficial.

However, immobilized enzymes showed lower activity in comparison to free enzymes. The possible reason may include their accessibility to substrate or any alterations induced during immobilization that causes structural changes in the enzyme molecule or from the creation of a microenvironment in which immobilized enzyme works. It is very likely that the characteristics exhibited by a free enzyme may be different than when they are attached to any carrier.

The physical and chemical properties of enzymes may undergo changes during the process of immobilization. Several factors such as temperature, pH, K_m, nature of the medium, product formation influences the rate of an enzyme catalyzed reaction. The microenvironment generated on account of enzyme-carrier interactions will most likely affect the above-mentioned factors as well as stability of enzyme, conformational and steric effects of enzymes.

13.2.1 Properties of Immobilized Enzymes

The attachment of an enzyme to an inert surface may impose certain behavioral changes. These changes will be reflected in the physical and chemical properties of immobilized enzymes. Major changes seen in the immobilized enzymes on account of microenvironment of the supporting matrix may be in their stability, conformational and steric effects or kinetic properties of the enzymes in comparison to the native enzyme.

Stability of Enzymes

There could be an increase or decrease in the stability of immobilized enzymes. The physical environment of support medium or its chemical nature provides a denaturation or stabilization atmosphere to the enzyme. Generally, the enzymes stability is enhanced on immobilization as the inactivation of enzymes due to autodigestion of proteolytic enzymes is significantly reduced. It has been observed that enzymes immobilized to inorganic carriers are more stable than those to organic polymers when stored at 4 or 25^{o}C.

Conformational and Steric Effects of Enzymes

As you know that most enzymes are proteins. Their tertiary structure will be affected on account of their immobilization to the supporting medium as determined by the spectral, fluorescence and circular dichroism studies on the enzymes during immobilization. In any enzyme reaction, the catalytic site holds key to the formation of products. Under ideal conditions, the immobilization process should not affect the catalytic site of an enzyme. However, an effect on the active site of enzyme during immobilization cannot be ruled out and these expected changes may arise due to change in the conformation of enzyme. As you know, enzyme interacts with specific substrate to form a transition state complex (ES) which is crucial for the product formation. The changes on account of crowding of atoms will lead to steric hindrance and may hamper the product formation. Therefore, an utmost caution is required in any immobilization process as well as the type of supporting matrix material on which the enzyme is immobilized.

Microenvironment of the Supporting Medium

The supporting medium of the immobilized enzyme influences its stability. The very presence of charged amino acids in the enzyme exhibiting an electrostatic effect along with bulk hydrophobic or hydrophilic groups present in the supporting matrix material may lead to desorption of the immobilized enzyme. The interactions of the microenvironment of immobilized enzyme with the substrate molecules also holds key to the product formation. Therefore, right choice of supporting matrix may provide the enzyme more stability.

Effect of pH on the Immobilized Enzyme

The pH of the immobilized enzyme is determined by the charged nature of its carrier (supporting matrix). If it is polyanionic (negative), there will be flow of H^+ ions towards it and if it is polycationic the flow of H^+ ions will be reduced. The pH optima of the immobilized enzyme will be low (acidic) when the carrier is polyanionic and it will be raised when the supporting carrier is polycationic.

SAQ 1

Do as directed:

a) The attachment of an enzyme to an inert surface may impose certain behavioral changes (True/False)

b) Enzymes get inactivated due to autodigestion by proteolytic enzymes (True/False).

c) The pH optima of the immobilized enzyme will be low/high when the carrier is polyanionic (Pick the correct option)

13.2.2 Types of Immobilization

There are number of ways by which enzymes can be immobilized. Entrapment method involves localization of enzyme in a polymer network. It is also considered as a type of physical adsorption. Enzyme does not bind with the carrier (support matrix). Therefore weak Vander waals interaction between enzyme and carrier causes little damage of enzyme activity.

A) Entrapment: a) Gel entrapment

b) Fibre entrapment

c) Microencapsulation

According to the binding mode of immobilization methods they can be classified by four ways. In physical methods weak interfaces between support and enzyme are present and in chemical methods covalent bonds are formed between the support and the enzyme.

B) Binding: a) Carrier binding

- Physical adsorption
- Ionic binding
- Chelation or metal binding
- Covalent binding

C) Cross-linking

Entrapment method:

These methods are generally used for cell immobilization but now-a-days these methods have been used for enzyme immobilization. In this method, enzyme is not bound to the polymer; however, its open flow is restrained or in other words simply caging of the enzyme. Entrapment methods are cheap and fast. Three types of entrapment methods are presently employed for enzyme immobilization. The commonly used method of enzyme is gel entrapment.

Gel Entrapment: Enzymes are entrapped within the interstitial spaces of cross linked water insoluble polymer gels. This method involves polymerization of monomer acrylamide molecules in an aqueous solution along with the soluble enzymes. Cross linking agents such as N, N-methylene polyacrylamide are used along with the usual initiators such as TEMED, ammonium persulfate or riboflavin for polymerization. The gel block is dispersed into particles of desired shape. The quantity of enzyme in surplus of 1 g of enzyme per gram of gel may be trapped. Table 13.1 enlists gels widely adopted for the entrapment of enzymes. The gelatinous nature of entrapment may lead to leakage of enzyme from the gels.

Table 13.1: Gel for the enzymes entrapment

S.No	Nature of Gel	Entrapped Enzyme
1	Polyacrylamide	Acetyl cholinesterase Chymotrypsin Asparaginase Glucose Dehydrogenase
2	Ethylene Glycol	Fructo Furanosidase Glucose Isomerase

Fibre Entrapment: The membrane confinement of enzymes may be realized by several methods which depend for their usefulness on the semi-permeable makeup of the membrane. These semi-permeable membranes while retaining the enzyme must allow free passage to the products and substrates. Fibre membranes are used for enzyme entrapment via non-covalent interactions. The high surface area, anti-microbial activity provided by thin fibres as well as resistance to weak acids and alkalis makes them better support material for enzyme immobilization. Hollow fibre membrane units are available commercially with large surface areas comparative to their controlled volumes (> 20 $m^2 L^{-1}$). These membranes are porous only to substances of molecular weight considerably smaller than the enzymes. Cellulose acetate in methylene chloride is used for enzyme entrapment. More than one enzyme can also be immobilized in the fibres. Several enzymes such as aminoacylase, glucoamylase, D-glucose isomerase, penicillin amidase and urate oxidase can be immobilized on cellulose acetate.

Microencapsulation: Spherical semi-permeable polymer membranes are used for microencapsulation of enzymes. The enzymes are enclosed by polymer membranes of diameter 1-100 µm. The enzyme immobilization engages Vander Waals and hydrophobic interactions. Substrates as well as products can diffuse across these polymer membranes provided they are smaller in size. This enzyme immobilization method is advantageous as it offers more surface area. In the interfacial microencapsulation method of enzyme immobilization, enzymes and hydrophilic monomer are emulsified in water immiscible organic solvent by stirring for example nylon encapsulate β-D-galactosidase or asparaginase. Liquid drying method of encapsulation involves dissolution of polymer in a water immiscible organic solvent at a boiling point lower than that of water. Thereafter aqueous solution of enzyme is dispersed in the organic phase to form water in oil emulsions that are further dispersed in aqueous phase having colloidal substances such as gelatin and surfactant forming a second emulsion. Vacuum removes the organic phase and a polymer membrane is produced to give enzyme microcapsules. Triacylgylcerol lipase and catalase enzymes can be encapsulated by ethylcellulose using this technology. Similarly, polystyrene is used to trap urease and triacylglycerol lipase enzymes.

Biological membranes such as liposomes are also well known for enzyme microencapsulation. Liposomes are artificially-prepared vesicles which are primarily composed of a lipid bilayer surrounding the soluble enzyme.

Binding: Binding methods are better than other immobilization techniques of localization because physical nature of binding between the enzyme and the carrier molecule may involve combinatorial effect of several non-covalent interactions such as hydrogen, hydrophobic, ionic and Vander Waals. Multiple interactions help the immobilized enzyme to withstand harsh conditions prevailing in the system. It is quite likely that the binding between enzyme and its support material may also leads to inactivation of enzyme or it may render the enzyme unusable.

Carrier Bound Enzymes: The properties of immobilized enzymes depend on the nature of enzyme as well as its support material for example substrate size, surface area of the carrier as well as chemical composition of the carrier. The interactions between the enzyme and carrier molecule influences the chemical, biochemical, mechanical and kinetic properties of the immobilized enzymes. The support (carrier) can be a synthetic organic polymer, a biopolymer or an inorganic solid and the binding mode may be physical, ionic or covalent. The carrier binding method can be further subclassified into physical adsorbtion, ionic binding, metal binding and covalent binding.

SAQ 2

a) Give two examples of enzymes immobilized on cellulose acetate.

b) Liposomes are also well known for enzyme microencapsulation (True/False)

c) Immobilized enzyme produced by micro encapsulation technique provides a small surface area (True/False)

d) The properties of immobilized enzymes do not depend on the nature of enzyme as well as its support material. (True/False)

Physical Adsorption: Physical adsorption of enzymes on water insoluble carriers relies on non specific interactions such as hydrophobic interactions, Vander Waals forces, hydrogen bonds between the enzyme and surface of the matrix. It is considered as the simple and economical method. This method causes little or no conformational change in the immobilized enzyme. Different processes involved in adsorption are static, electro deposition, reactor loading or mixing or shaking bath loading. For commercial purposes reactor loading is the most preferred method. The carrier is loaded first into the reactor followed by the addition of enzyme and then recirculation or agitation is done through the reactor. In electrodeposition, carrier is positioned next to one of the electrodes in an enzyme bath. On passing the current, enzyme migrates to the carrier and gets deposited on the surface. Physical adsorption is advantageous because relatively fewer steps of activation are required for enzyme immobilization but suffers on account of desorption of enzyme from the carrier due to changes in pH, temperature and ionic strength. Table 13.2 enlists immobilized enzymes along with their carrier used for physical adsorption.

Table 13.2: Enzymes immobilization by Physical Adsorption

S.No	Nature of Carrier	Immobilized Enzyme
1	Phenoxyacetyl cellulose	Alkaline Phosphatase
2	Palmitoyl cellulose	Triacylgylcerol Lipase Naringinase
3	Zeolite	Trypsin Chymotrypsin
4	Silica gel	Alcohol Dehydrogenase

Covalent Binding: As the name suggests and you can also guess that this method of enzyme immobilization involves covalent bond formation between the enzyme and support matrix. It is one of most important methods of immobilization techniques as the selection of conditions for immobilization is more difficult than the other methods. A major limitation being that the binding does not lead to loss of enzyme activity. On the other hand, major advantage lies in the strength of binding between enzyme and the solid support which prevents the leakage of enzyme even in the presence of substrate and solutions of high ionic strength. The mode of covalent attachment must involve functional groups of the enzyme that are not involved in the catalytic action. Numbers of protective methods are being adopted to prevent inactivation reactions with amino acid residues in the active site of enzymes. These methods include covalent attachment of enzyme in the presence of

competitive inhibitor or substrate, a reversible covalently linked enzyme inhibitor complex or a zymogen precursor.

The covalent binding can be done by several methods such as diazotization, amide bond formation, alkylation and arylation, Schiffs base etc (Table 13.3).

Table 13.3: Enzyme Immobilization by covalent bonding using different processes

S.No.	Name of Process	Chemical Procedure	Examples
1.	Process of diazotization	Support-N=N-ENZYME	β-D-Galactosidase D-Glucose oxidase Trypsin Papain Protein kinase Chymotrypsin
2.	Using Amide bond formation	Support-CO-NH-ENZYME	Alkaline phosphatase Naringinase Trypsin Chymotrypsin Papain Penicillin amidase Papain
3.	Alkylation and Arylation method	Support-CH_2-NH-ENZYME Support-CH_2-S-ENZYME	Aminoacylase Glucoamylase Trypsin Amylase
4.	Schiff's base formation method	Support-CH=N-ENZYME	Penicillinase β-D-Galactosidase D-Glucose oxidase Trypsin
5.	Amidation reaction process	Support-CNH-NH-ENZYME	
6.	Carrier binding with	Support-	

	bifunc- tional reagents	$O(CH_2)_2N=CH(CH_2)_3$ CH=N-ENZYME	

Eupergit-C (Fig. 13.1) is an acrylic resin and a copolymer of N, N'-methylene-bi-metacrylamide and glycidyl methacrylate. Immobilization by covalent binding to Eupergit-C has been successfully used for a variety of industrially applied enzymes for example penicillin amidase. The average particle size of Eupergit-C is 170 µm with pore diameter of 25 nm. It is highly hydrophilic and stable over a wide range of pH (0-14). It binds to proteins at neutral or alkaline pH.

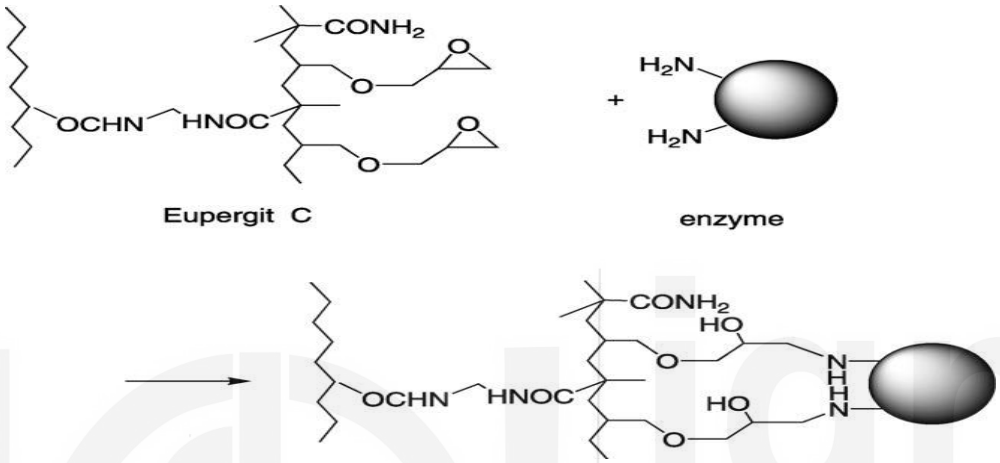


Fig. 13.1: Immobilization of enzyme on Eupergit-C.

Ionic Binding: The ionic method of binding involves ionic interactions between side chains of enzyme (lysine, arginine, histidine, aspartate and glutamate) with water insoluble carriers containing ion exchange residues. Ionic interactions provide strength to the enzyme carrier linkages. Protein macromolecules and polysaccharides such as gelatin, albumin cellulose, dextran, starch and chitosan are extensively used biopolymers for immobilizing enzymes. Ionic method is similar to the ion-exchange chromatography where a diffusible exchanger ion is attached to that of inert immovable counter ion exchanger. This method causes little changes in the conformation or active site of enzyme and therefore immobilized enzymes are of high reactivity. The most common anion exchanger is DEAE (diethyl amino ethyl) derivative while CM (carboxy methyl) derivatives are the most common cation exchanger (Table 13.4). Aminoacylase from Aspergillus oryzae immobilized on DEAE-Cellulose is used for the production of L-amino acids. D-Glucose isomerase and beta-D-fructofuranosidase can also be immobilized on DEAE-Sephadex. Similarly, immobilized biocatalyst epoxide hydrolase from Aspergillus niger is active at high substrate concentration and can be recycled seven times.

Table 13.4: Enzyme Immobilization by Ionic bonding

S.No.	Inert Carrier	Exchanger	Examples of Immobilized enzymes
1.	DEAE-Cellulose	Anionic	Glucoamylase Phosphodiesterase D-Glucose Isomerase Aminoacylase
2.	AE- Cellulose	Anionic	D-Glucose Isomerase
3.	DEAE-Sephadex	Anionic	D-Glucose Isomerase beta-D-fructofuranosidase
4.	CM-Cellulose	Cationic	Pencillin Amidase Amino acyl –tRNA Synthetases
5.	CM-Sephadex	Cationic	Dextran Sucrase

Chelation or Metal Binding: Immobilization of enzymes can be carried out by transitional metal compounds. They are used to activate the surface of inert solid matrix for direct coupling with the enzyme through the formation of chelates. Several inert supports used in this process are glass, chitin, alginic acid and gelatin. You can see the table 13.5. It enlists several enzymes immobilized by transition metals. Transition metals involved in this type of enzyme immobilization include titanium (III) and zirconium (IV) that form polymeric oxides and hydroxides.

Table 13.5: Enzyme immobilization by metal binding

S.No.	***Nature of Support***	***Enzyme Immobilized***
	a) *Organic Supports*	
1.	DEAE- cellulose	Pectin lyase
2.	Nylon	D-Glucose dehydrogenase
3.	Cellulose	β-D-Fructofuranosidase
	b) Inorganic Supports	
4.	Alumina	Glucoamylase
		β-D-Xylosidase

5.	Magnetic iron oxide	α –Amylase
6.	Silica gel	Trypsin
7.	Titanium (IV) oxide	Dextranase
8.	Hydrous metal oxides prepared from Ti (IV), Zr(IV), Fe(III) and Sn(II)	Chymotrypsin Glucoamylase D-Glucose oxidase Papain Peroxide Trypsin

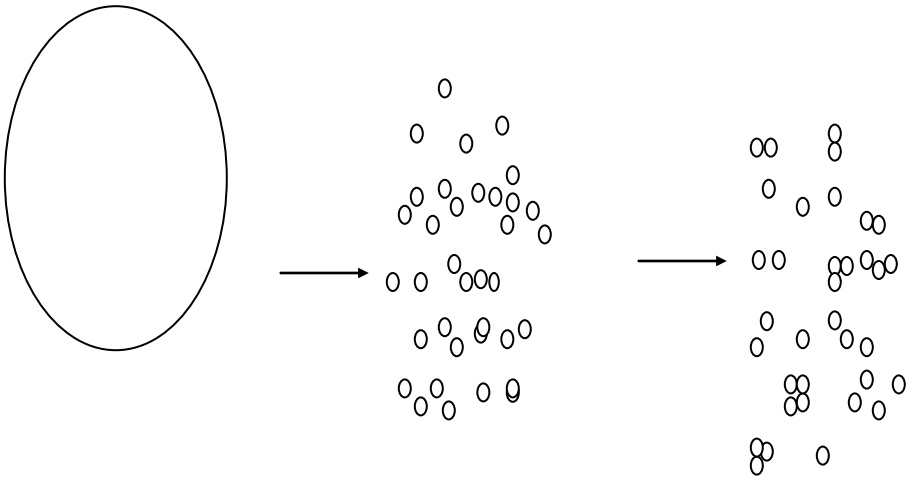
Cross linked Enzymes: Immobilization by cross linking of enzymes can be achieved by intermolecular cross linking (formation of covalent bonds between enzyme molecules by means of cross linking agents) to form three dimensional cross linked aggregates with functional groups on an insoluble support matrix. It is not an ideal method since many molecules simply act as support to others. Therefore, cross linking is performed in conjunction with other methods of enzyme immobilization. The reagents required for cross linking generally has two identical functional groups or two or more functional groups. Some of the commonly used reagents are

a) Carboxy functional groups react with L-Lysine residues by Schiffs base formation

b) Diazo groups reacts with L-Lysine, L-Histidine, L-Tyrosine or L-Cysteine

c) Isocyanate groups react with amide (peptide) bond formation

The most common reagent used for cross linking is glutaraldehyde. The process of cross linking occurs via glutaraldehyde and NH_2 groups present on the surface of enzyme for example cross linking of carboxypeptidase A with glutaraldehyde. Cross linking by glutaraldehyde process has several disadvantages such as low activity retention, poor reproducibility and low mechanical stability. The process of enzyme crystallization is laborious and time consuming. Moreover, purification of proteins involves addition of salts, water miscible organic solvents or non-ionic polymers. Retention of enzyme bioactivity on crystallization is of immense value that led to the new family of immobilized enzymes: cross linked enzyme aggregates (CLEA) [Fig. 13.3]. Industrially important enzymes such as penicillin amidase CLEA are effective catalysts for the synthesis of penicillin. Similarly, CLEAs of several commercially important enzymes such as lipases have shown excellent performance in water than their standard immobilized form.



Free Enzyme in Solution (5 nm) | Aggregate (1 um) | CLEA (1-100 um)

Fig. 13.3: Preparation of a CLEA.

SAQ 3

Do as directed:

a) The immobilized technique involving chemical method is covalent dependent/non-covalent dependent.

b) In adsorption method of enzyme immobilization, the state of immobilization is very sensitive to solution ……., ……. and …….

c) ……. is the most commonly employed cross linked polymer.

d) The most common reagent used for cross linking is glutaraldehyde (True/False)

13.3 ENZYME ENGINEERING

Since you know enzymes are proteins so the term enzyme engineering is synonyms with protein engineering. The procedure of customizing a new enzyme with improved properties by changing or transforming the amino acids in the enzyme is known as enzyme engineering. These improved properties may include the following characteristics:

a) Kinetic properties of enzyme turnover and Michaelis Constant K_m

b) Thermostability and optimum temperature for the enzyme

c) Stability and activity of enzyme

d) Substrate and reaction specificity

e) Stability and activity of enzyme

f) Substrate and reaction specificity

g) Cofactor requirements

h) Optimum pH

i) Protease resistance

j) Allosteric regulation and

k) Molecular weight and subunit structure

The changes may include combination of some of these above mentioned characteristics to get the most efficient form of enzyme. The general approaches in enzyme engineering include rational designing, semi-rational design, DNA shuffling, structure base design and directed (molecular) evolution, random mutagenesis, cell surface display technique, etc. We will discuss the three main strategies for protein engineering i.e. directed evolution, rational protein design, and semirational design.

Directed Evolution

Directional evolution is a new approach of enzyme engineering, which includes random mutagenesis and high throughput screening (HTR). In this method gene is subjected to multiple rounds of mutagenesis (creating a library of variants) followed by selection (expression of those variants and isolation of ones with the preferred function) further followed with amplification (production of a pattern for the subsequent rounds).

The engineering could be accomplished by three major methods: (1) randomly recombining a set of related sequences (e.g., DNA shuffling) to allow recombination between existing genes for related enzymes. This technique is used to develop drugs, dyes and fragrances. Enzymes can be produced in large numbers using this technique. (2) Introduction of random changes in single protein sequences (point mutations by chemical mutagens or error prone PCR) or insertions and deletions (using transposons); this method involves mutagenesis and selection for improving the specific property of enzyme. Example includes Xanthine dehydrogenase enzyme that oxidizes 2 hydroxy purine at position 8 but a mutant shows oxidation at position 6 of 2 hydroxypurine. Lactate dehydrogenase can be modified to malate dehydrogenase by a natural mutation leading to single amino acid substitution (Gln^{102}-Arg) (3) screening of coding genes. Each coding gene is being expressed and assayed for the quantitative measurement of the activity

Most of the mutations are lethal therefore libraries of mutants tend to have variants with reduced activity. High-throughput assays is required for measuring activity to find the exceptional variants with valuable mutations that has the desired or improved properties. Two types of method are used for the detection and isolation of functional variants. Selection systems directly pair protein function to gene survival, whereas screening systems individually assay each variant and permit a quantitative threshold to be set for sorting a variant or population of variants of a desired catalytic activity. Both selection

and screening can be performed in living cells (*in vivo* evolution) or carried out directly on the protein or RNA without any cells (*in vitro* evolution)

It is necessary to know the detailed information of protein, such as the amino acid sequence, structural, and the active site, and the thereafter the scope of application could be expanded. However procedure of directed evolution would obtain huge number of mutants, which need to be further screened by several rounds. Hence, this procedure is time consuming and laborious for further high-throughput assays.

Rational Protein Design

Several important developments such as availability of database of protein structure, sequences and functions have made it possible to arrange proteins in family trees and to understand their functions. These developments are proving very helpful for protein engineering. In the rational design process, amino acid sequences are mutated by specific blueprint based on the comprehensive information of the protein (structure function relationship) which is applied by mutations using site directed mutagenesis to the change the specific amino acids of the protein through substitution, insertions, or deletions. Rational protein design process includes recombination-dependent exponential amplification PCR, homologous recombination, and point mutation. Lately rational protein design has been applied to improve the catalytic activity and stability of natural enzyme protein, substrate specificity, coenzyme specificity, enantioselectivity, expression level and new catalytic functions. The rational design is universal, fast and also one of the highly successful enzyme engineering methods, but the design relies on the profound understanding of the structure-function relationship of protein structure. This method has the prospective to be developed into algorithms that can quantitatively envisage the act of the designed sequences. Important examples include engineering of membrane bound di-iron enzymes having activities like hydroxylase, epoxidase and conjugase activities during fatty acid biosynthesis in plants. Four amino acid substitutions convert a desaturase to hydroxylase and six substitutions convert a hydorxylase to desaturase

Semirational Design

This type of protein engineering method coalesce the benefits of directed evolution with rational protein design to generate smaller libraries based on the known information of protein (biochemical, structural data, etc.). Semi-rational approaches of enzyme engineering target numerous precise amino acid residues to mutate on the basis of previous available structural or functional information to produce 'smart' libraries that are more likely to give positive results. The approach is highly beneficial when no HTS is available. In such a scenario a smart library can be built with same number of mutants than with a random whole gene mutagenesis. Experimental and computational methods offer efficient sampling of mutations that can affect enzyme function with remarkable improvements in substrate selectivity and specificity and in the de novo design of enzyme activities within scaffolds of known structure. The combinatorial active-site saturation test (CAST) is one of the extensively used technology for the semirational design that employs the information from

structural data to identify the specific amino acids (e.g., active site) for mutation by random mutation or/and site-saturation mutagenesis.

Gene Fusion

In this technology, polyfunctional muti enzymes can be generated by joining of structural genes or two or more enzymes. The translational stop signal at 3' end of first gene is removed and ligated in frame to ATG start codon of the second gene. Short linkers can also be used sometimes. The novel chimaeric gene has a single polypeptide chain carrying active sites of both genes. The gene fusion may involve a) monomeric enzymes b) monomeric and dimeric enzyme c) two dimeric enzymes. Examples include gene fusions of galactosidase-galactokinase, galactosidase-galactose dehydrogenase (Fig.13.4).

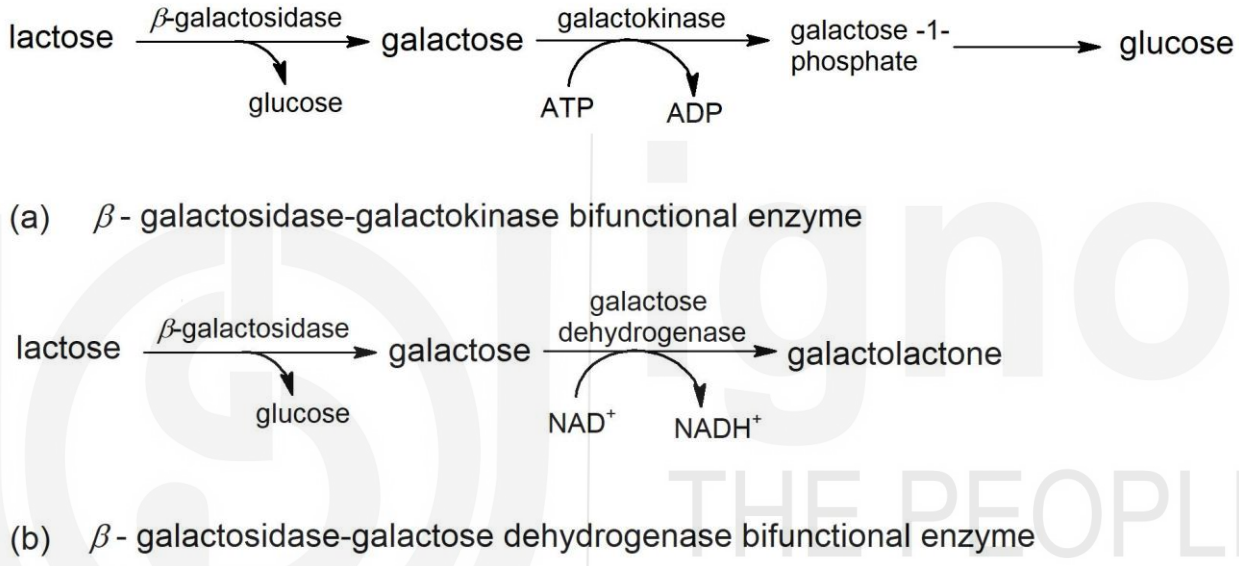


Fig. 13.4: Reactions catalyzed by bifunctional enzyme constructs a) galactosidase-galactokinase b) galactosidase-galactose dehydrogenase.

In conclusion, enzyme engineering has been developed as a promising tool and technology to improve the properties of native enzymes and plays an important role in the application of industry.

Chemical modification of enzymes

We all know that protein can undergo post translational modifications. In proteins or enzymes such modifications can causes change in stability, integrity, altered solubility and reactivity. The in vitro alterations may sometimes be able to generate variant enzyme or entirely new enzyme by creating new active sites or altering the existing ones. Some of the examples are:

a) Enzyme-PEG conjugates: Enzyme L-asparaginase is toxic with active for only 18 hrs. However, L-asparaginase from E.Coli can be altered by polyethylene derivatives to generate PEG-asparaginase conjugates. These conjugates differ from original enzyme as they retain 52 % of catalytic activity, resistance to proteolytic degradation and the variant enzyme does not cause allergy. So, this enzyme is more useful to treat malignant tumors than the native enzyme.

b) Alteration of proteases to peptide ligases: Peptide ligation to native enzymes leads to high specificity and stereoselectivity and suppress side reactions.

c) Production of site specific nucleases: Fusion of non-specific phosphodiesterases to oligonucleotides of defined sequence can generate site specific nucleases. This approach can be used for the production of artificial restriction enzymes.

Hybrid Enzymes

Genetic engineering approaches have made it feasible to generate hybrid enzymes. In these enzymes, each enzyme holds portions of two or more enzymes thus increasing the prospective uses of natural enzymes. The alterations include non-catalytic and catalytic properties of enzymes to produce hybrid enzymes. These novel enzymes are produced by

1) Single point mutation or by substitution of secondary structure from one enzyme to other enzyme

2) Transfer or exchange of a secondary structure element or a monomeric unit of multimeric enzyme with other enzyme

3) Fusion between two enzymes having separate and distinct activities.

Hybrid enzymes are being used for biosynthesis of group of secondary metabolite called polyketides acquired through repeated condensation of acetyl and malony units to produce compounds like antibiotics, anti cancer agents or immune suppressants.

Artificial enzymes

Molecules designed and modified to display enzyme-like characteristics are called as artificial enzymes. These enzymes have a substrate-binding site and a transformation site that are employed for fast binding and conversion of substrate molecules into products. They may mimic the catalytic ability and specificity of a well-characterized enzyme or catalyze non-naturally occurring chemical reactions using known mechanisms of enzymatic catalysis. The benefits of artificial enzymes are that they show faster reaction rate constants in comparison to natural enzymes while displaying similar catalytic activity. They are more flexible and effective over a range of environment conditions such as pH, temp, pressure or ionic strength. Widespread method utilized in the production of artificial enzymes involves addition of an adequate functional group to macromolecules so that they can mimic the amino acid residues in the active site of enzymes.

Classically, artificial enzymes bind substrates using receptors such as cyclodextrin, crown ethers, and calixarene. Artificial enzymes based on amino acids or peptides have increased the field of artificial enzymes or enzyme mimics. For example, scaffolded histidine residues mimic certain metalloproteins as well as enzymes such as hemocyanin, tyrosinase, and catechol oxidase. Some of different types of enzyme mimics are:

Chemzymes: These are enzyme mimics that employs supramolecular structure and artificial, non-protein molecules as host molecules. The examples include cyclodextrin-based artificial enzymes and diaminoxanthone-derived artificial enzymes.

Synzymes are the synthesized enzymes made from naturally occurring host molecules such as oligomers, polymers, and derivatized proteins.

Nanozymes: Nanozymes are nanomaterials based artificial enzymes having enzyme-like characteristics. They have been explored for applications such as biosensing, bioimaging, tumor diagnosis and therapy, and anti-biofouling. Examples include magnetoferritin (MFt) nanoparticles that mimic peroxidase activity.

Achievements of Enzyme Engineering: Some of the achievements of enzyme engineering are as listed below:

1) T4 Lysozyme: The change in 51st threonine into a proline increases the affinity of T4 lysozyme as well its activity by 25 times.
2) Enzyme xylanase from Thermomyces lanuginosus by error-prone PCR led to an increase in its half life from 9 to 25 min under 100 C .
3) A phenylalanine residue has been involved in the reducing potential of protein Cytochrome C in electron transport chain but non essential for electron transfer.
4) Trypsin can be redesigned with altered substrate specificity
5) Replacement of single amino acid aspartic acid to asparaginase decreases specific activity of Dihydrofolate reductase.
6) $T_{1/2}$ of lipase from Candida antarctica enhances from 2 to 24 h under 45°C after DNA shuffling.

SAQ 4

Do as directed:

a) Name two approaches for enzyme engineering.

b) This type of enzyme engineering method combines the benefits of directed evolution with rational protein design. Name the method.

c) Enzyme-PEG conjugate is an example of chemical modification of enzyme (True/false).

d) Nanozymes are nanomaterials (True/False)

13.4 SUMMARY

Let us summarize:

- The extensive range of commercial application of enzymes has led to strong focus on the immobilization processes. The usage and development of advanced techniques of enzyme isolation and purification has also paved their way of immobilization to several mediums.
- It is necessary to understand that changes in the physical and chemical properties of enzymes are expected during immobilization. The stability of enzymes may also be affected. The enzyme-supporting matrix interactions generated microenvironment may influence the conformational and steric effects of enzyme.
- There are many types of immobilization methods and several parts of an enzyme molecule are targeted for immobilization. Various gels such as polyacrylamide and ethylene glycol are used for entrapment of enzymes.
- A high surface area is provided by fibre entrapment method for enzyme binding. Microencapsulation is another method for the immobilization of enzyme. Either individually or combinatorial manner, non–covalent interactions such as hydrogen, hydrophobic, ionic are used to immobilize the enzyme. Carrier binding method of enzyme immobilization is further classified into physical adsorption, ionic binding, metal binding or covalent binding. Intermolecular cross linking with groups on support matrix also helps in enzyme immobilization.
- Many natural enzymes generally show some limitations, such as low catalytic efficiency, low activity, and low stability, etc. These factors normally limited the application of these wild-type enzymes. Enzyme engineering could improve the properties of enzyme, and the modified enzyme could be applied broadly. Several methods of enzyme engineering can be used individually or in combination.

13.5 TERMINAL QUESTIONS

1. What do you understand by statement “Liposomes are well known for enzyme microencapsulation”. Explain the concept of microencapsulation.
2. Discuss ionic binding and covalent binding methods of enzyme immobilization?
3. Elaborate on different types of immobilization by entrapment.
4. How cross-linked enzymes are formed? Explain with diagrams.
5. Discuss different types of enzyme engineering.

13.6 ANSWERS

Self-Assessment Questions

1. a) True b) True c) low
2. a) Aminoacylase, Glucoamylase, D-glucose isomerase, pencillin amidase and urate oxidase b) True c) False d) False
3. a) covalent dependent, b) pH, ionic strength and temperature, c) polyacrylamide gel d) True
4. a) rational designing, semi-rational design, DNA shuffling, b) Semirational Design c) True d) True

Terminal Questions

1. Refer to microencapsulation section of 13.2.2
2. Refer to covalent and ionic bonding sections of carrier bound enzymes.
3. Refer to section A of 13.2.2
4. Refer to section on cross linked enzymes.
5. Refer to section 13.3

13.7 FURTHER READINGS

1. David L. Nelson and Michael M. Cox: Lehninger Principles of Biochemistry 6th Ed., W.H. Freeman.
2. Robert K. Murray, Daryl K. Granner, Victor W. Rodwell Harper's Illustrated Biochemistry, 27th edition. 2006, McGraw-Hill.
3. Donald J Voet and Judith G. Voet:Principles of Biochemistry4th ed., John Wiley and Sons, Inc, USA.
4. Eric E Conn, Paul K Stumpf: Outlines of Biochemistry, Jophn Wiley and Sons, Inc, USA.
5. S. Shanmugan and T. Sathishkumar: Enzyme Technology, I K International Publishing House Pvt Ltd, New Delhi.
6. Nicholas C Price and Lewis Stevens: Fundamentals of Enzymology, Oxford University Press, Oxford, New York, USA.

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