Unit 1: Enzyme Biotechnology, Biosensors, Protein Engineering and uses of microorganisms in the production of enzymes.

6th Sem B.Pharm
Pharmaceutical Biotechnology

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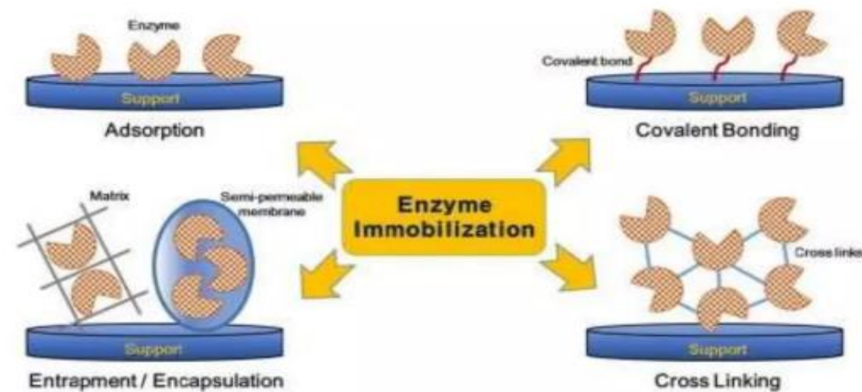
ENZYME BIOTECHNOLOGY -
Methods of Enzyme Immobilization & Its Applications



What Is Enzyme Immobilization ?

Enzyme immobilization may be defined as a process of confining the enzyme molecules to a solid support over which a substrate is passed and converted to products. The process whereby the movement of enzymes, cells, organelles, etc. in space is completely or severely restricted usually resulting in a water-insoluble form of the enzyme

What Is An Immobilized Enzyme?

An immobilized enzyme is one whose movement in space has been restricted either completely or to a small limited region.

Need for Immobilization

- Protection from degradation and deactivation.
- Recycling, repetitive use.
- Cost efficiency.
- Enhanced stability.
- Use as controlled release agents.
- The ability to stop the reaction rapidly by removing the enzyme from the reaction Solution (or vice-versa)
- Allows development of multi-enzyme reaction system.

SALIENT FEATURES OF ENZYME IMMOBILIZATION

- The enzyme phase is called as carrier phase which is water insoluble but hydrophilic porous polymeric matrix, e.g. agarose, cellulose, etc.
- The enzyme phase may be in the form of fine particulate, membranous or microcapsule.
- The enzyme in turn may be bound to another enzyme via cross linking.
- A special module is produced employing immobilization techniques through which fluid can pass easily, transforming substrate into product and at the same time facilitating the easy removal of catalyst from the product as it leaves the reactor.

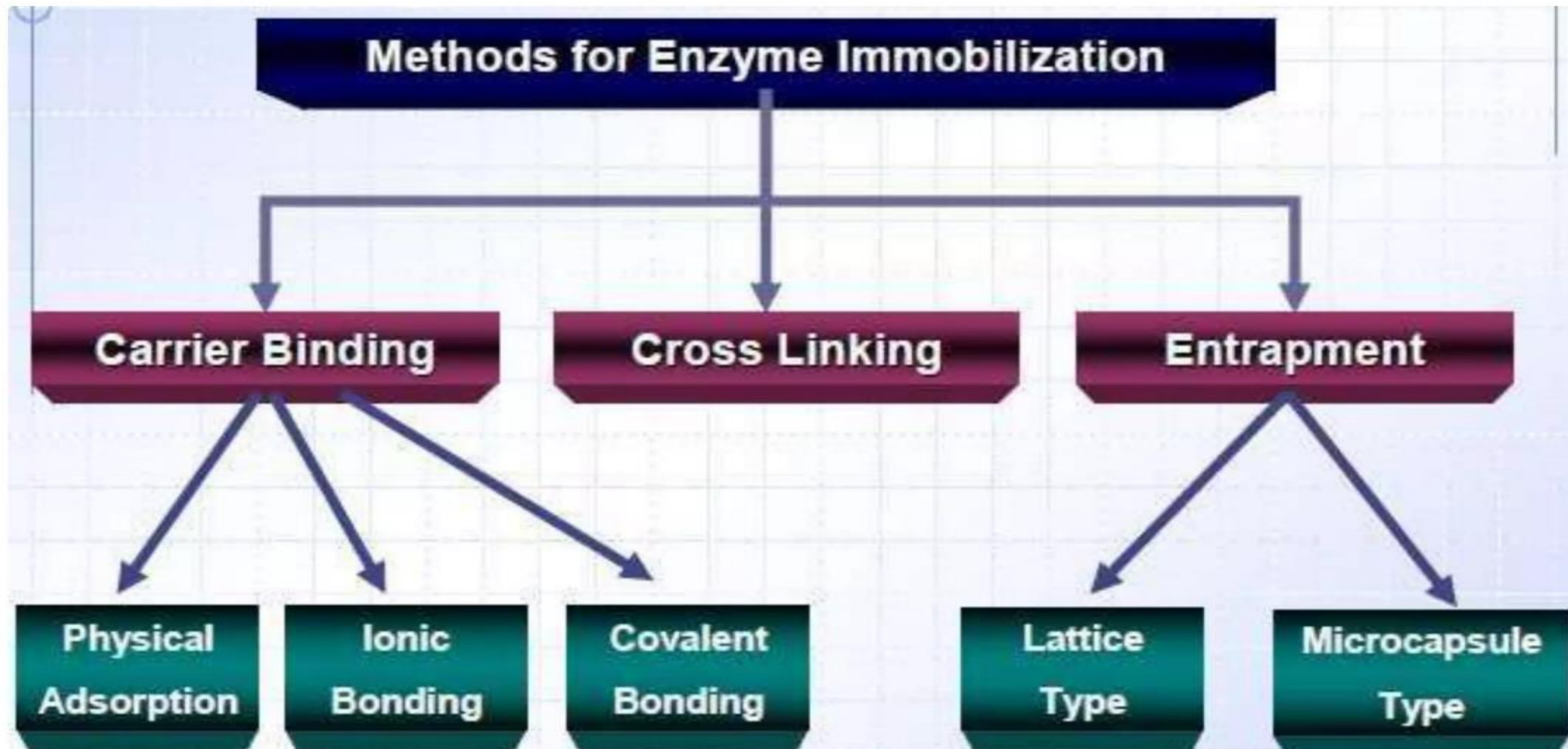
- The support or carrier utilized in immobilization technique is not stable at particular P^H, ionic strength or solvent conditions. Hence, may be disrupted or dissolved releasing the enzyme component after the reaction.

Advantages of enzyme immobilization:-

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

Disadvantages of enzyme immobilization:-

- It gives rise to an additional bearing on cost.
- It invariably affects the stability and activity of enzymes.
- The technique may not prove to be of any advantage when one of the substrate is found to be insoluble.
- Certain immobilization protocols offer serious problems with respect to the diffusion of the substrate to have an access to the enzyme.



Methods for Enzyme Immobilization
Carrier Binding
Cross Linking
Entrapment
Physical Adsorption
Ionic Bonding
Covalent Bonding
Lattice Type
Microcapsule Type

PHYSICAL ADSORPTION

This method is based on the physical adsorption of enzyme protein on the surface of water-insoluble carriers.

Examples of suitable adsorbents are ion-exchange matrices, porous carbon, clay, hydrous metal oxides, glasses and polymeric aromatic resins.

The bond between the enzyme and carrier molecule may be ionic, covalent, hydrogen, coordinated covalent or even combination of any of these.

Method of immobilization by adsorption

1. This method is based on the physical adsorption of enzyme protein on the surface of water-insoluble carriers.
2. The enzyme is adequately mixed with a right adsorbent under appropriate P^H & ionic strength.
3. After incubation for certain period of time, the carrier matrix is washed thoroughly to get rid of entire unadsorbed enzyme molecules whereby the immobilized enzyme is ready for actual usage.
4. This specific method gives rise to high loading (1gm enzyme/gm matrix) of enzyme.

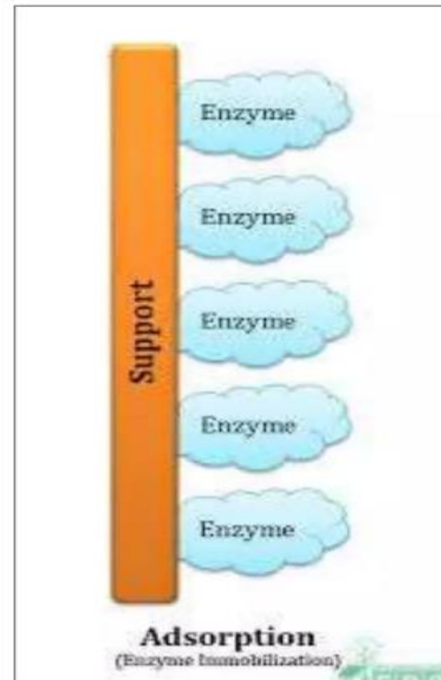
Advantages of adsorption:-

- Little or no confirmation change of the enzyme.
- Simple and cheap.
- No reagents are required.
- Wide applicability and capable of high enzyme loading.

Disadvantages of adsorption:-

- Desorption of the enzyme protein resulting from changes in temperature, P^H and ionic strength.
- Slow method.

CARRIER BINDING - PHYSICAL ADSORPTION



Enzymes immobilized by adsorption

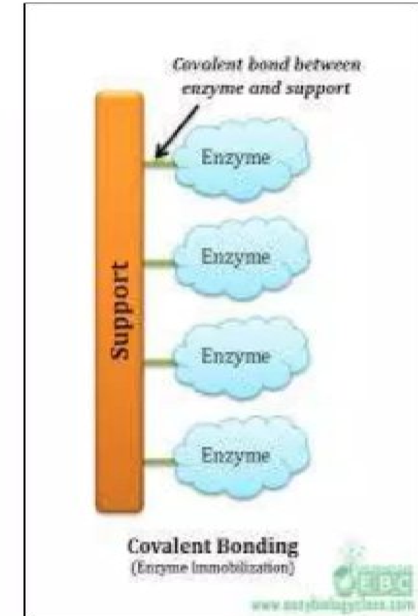
S.No	Name of the enzyme	Adsorbent
1	α-Amylase	Calcium phosphate
2	Amyloglucosidase	Agarose, DEAE-sephadex (Diethylaminoethyl)
3	Catalase	Charcoal
4	Glucose oxidase	Cellophane
5	Invertase	Charcoal, DEAE-sephadex

Covalent bonding

- Widely used
- The covalent bond between enzyme and a support matrix forms a stable complex. The functional group present on enzyme, through which a covalent bond with support could be established, should be non essential for enzymatic activity.
- The most common technique is to activate a cellulose-based support with cyanogen bromide, which is then mixed with the enzyme.

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

- Amino group
- Carboxylic group
- Phenol ring
- Indole group
- Imidazole group



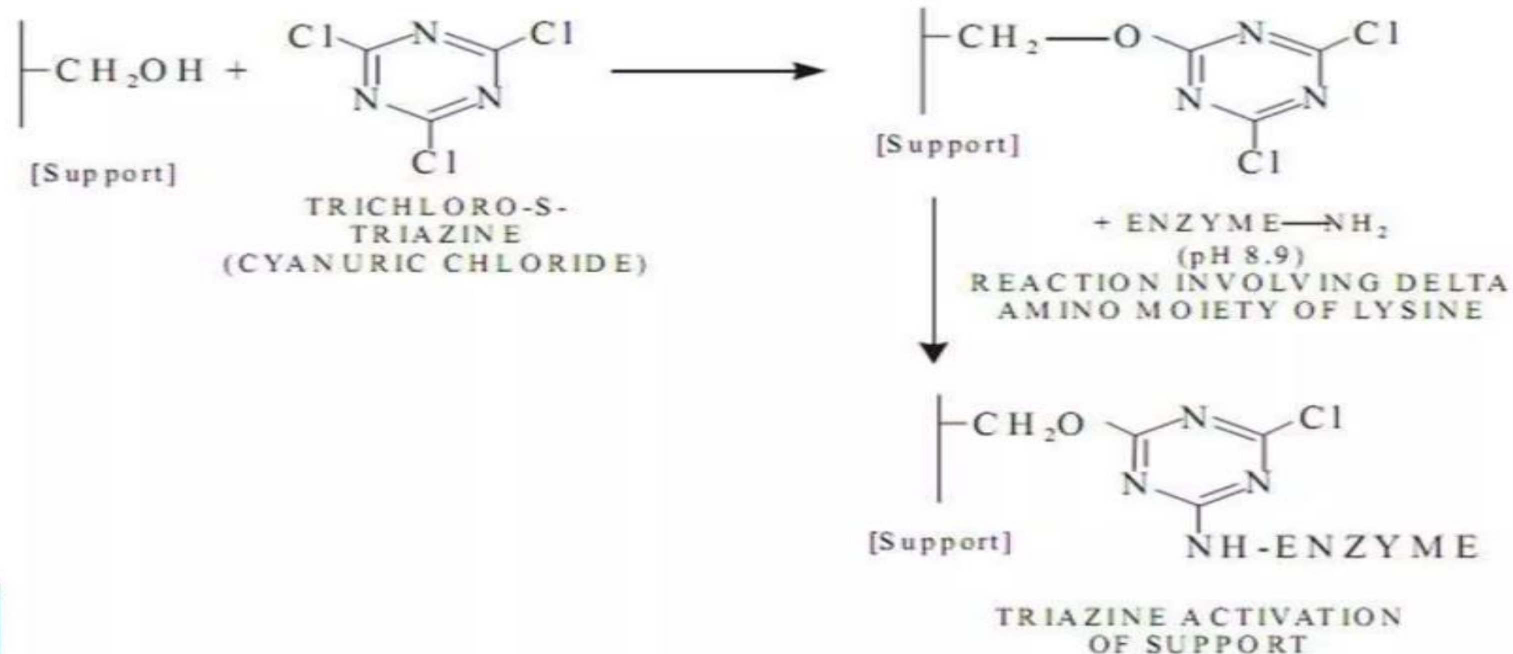
The most commonly used polymers are polysaccharides, polyvinyl alcohol, silica and porous glasses.

Enzymes immobilized by covalent bonding using carrier matrix & binding reactions are

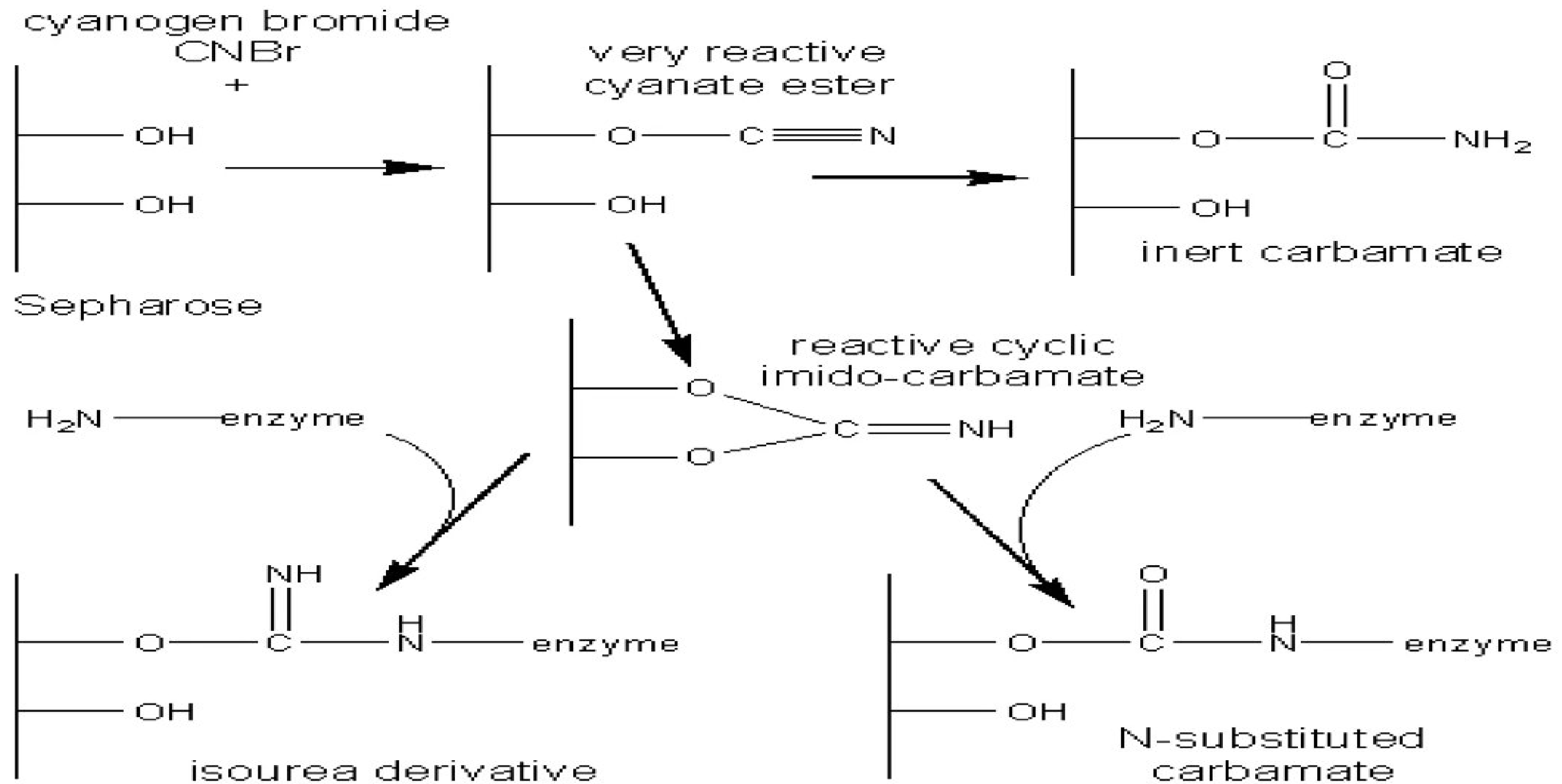
S.No	Enzyme	Carrier matrix	Binding reaction
1	α-Amylase	DEAE-cellulose	Direct coupling
2	Amyloglucosidase	DEAE-cellulose	Cyanuric chloride
3	Cellulose	Polyurethane	Isocyanate
4	Glucose isomerase	Polyurethane	Isocyanate
5	Pectinase	Polyurethane	Isocyanate
6	Glucose oxidase	Porous glass	Isothiocyanate

Covalent bonding

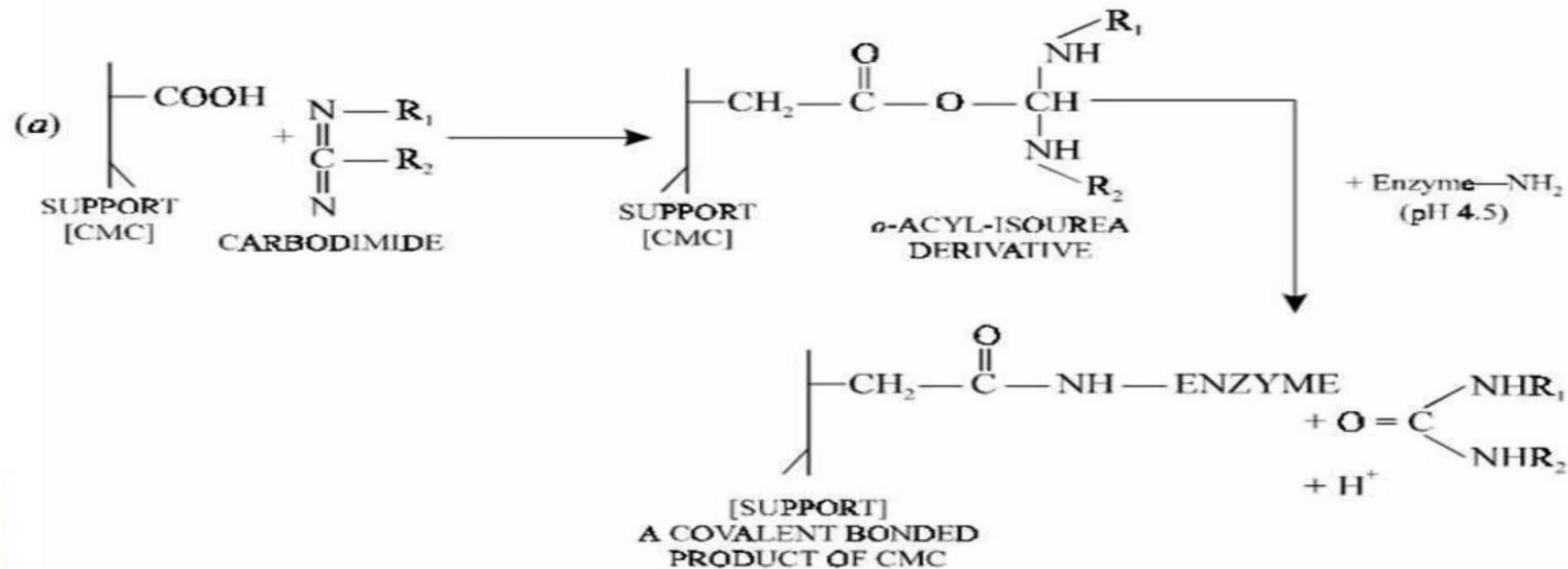
Using Supports with – OH group that are Activated by Covalent Bonding with Triazine.



- **Activation by CNBr**



- Immobilization of Enzymes using CMC Supports Having — COOH with — NH_2 Group or with Hydrazine (NH2-NH2) Group via Covalent Bondage Involving Acyl Urea .



Advantages of covalent bonding:-

- The strength of binding is very strong, so, leakage of enzyme from the support is absent or very little.
- This is a simple, mild and often successful method of wide applicability

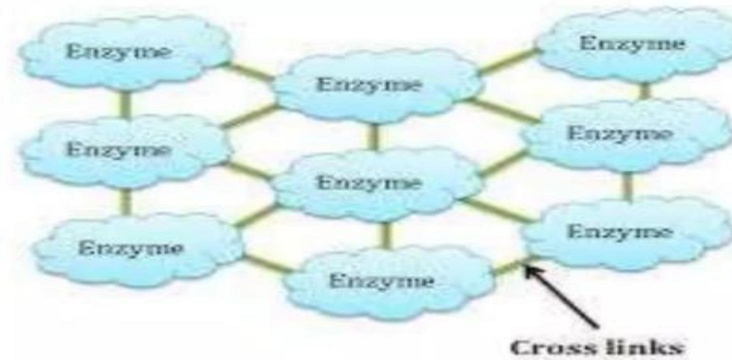
Disadvantages of covalent coupling:-

- Enzymes are chemically modified and so many are denatured during immobilization.
- Only small amounts of enzymes may be immobilized (about 0.02 grams per gram of matrix).

Cross linking

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

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



Cross linking

Advantages of cross linking:-

- Very little desorption(enzyme strongly bound)
- Best used in conjunction with other methods.

Disadvantages of cross linking:-

- Cross linking may cause significant changes in the active site.

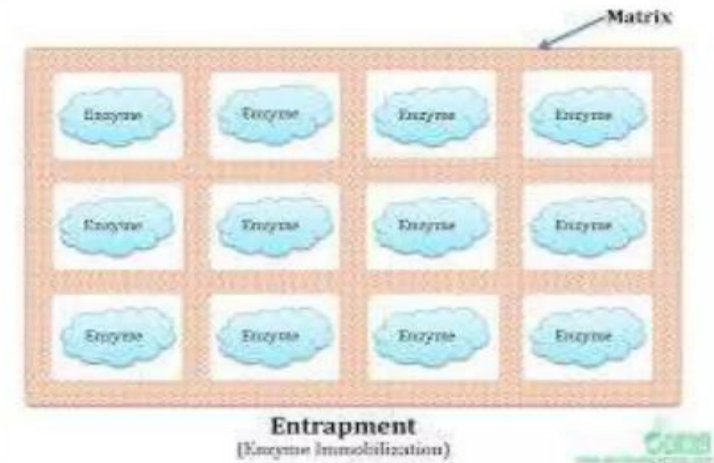
Entrapment

- The enzymes is trapped inside the polymer matrix.
- Biocatalyst + monomer solution

  ↓ Polymerization (Change in temp, P^H)

  Enzyme entrapment

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



ENTRAPMENT

- Entrapment in gel may cause:
  - Matrix polymerization or
  - Precipitation or
  - Coagulation
- Entrapment in calcium alginate is most widely used entrament for:
  - Microbial
  - Animals
  - Plant enzymes/cells

  Ex: Glucose oxidase + Polyacrlamide (entapment gel)

Advantages of entrapment:-

Loss of enzyme activity upon immobilization is minimized.

Disadvantages of entrapment:-

The enzyme can leak into the surrounding medium.

Another problem is the mass transfer resistance to substrates and products.

Substrate cannot diffuse deep into the gel matrix.

Reactors with Enzymes Immobilized on Membranes

Products

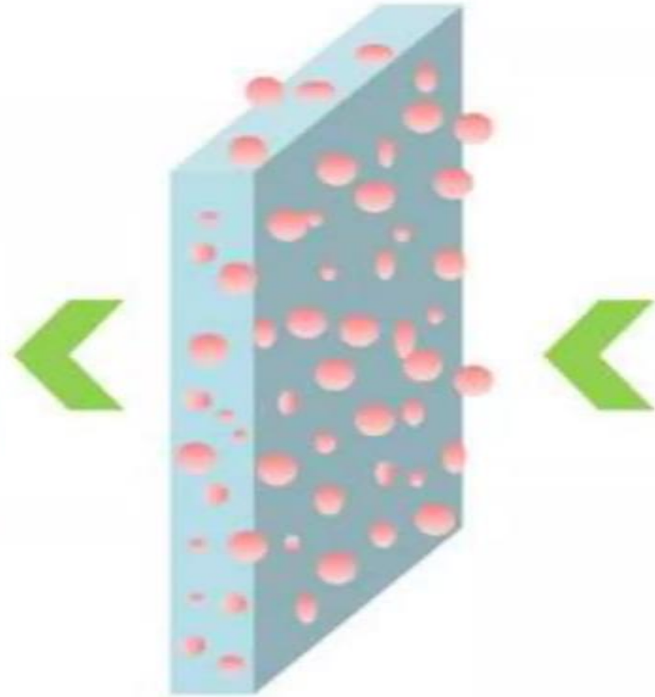
Polypeptides, cyclodextrins, low molecular weight proteins, Small molecule sugar, fatty acids, triolein, glycerin, *etc.*

or

Small molecule non-polluting substance.

or

L-Malic acid ,methanol, isomalto-oligosaccharides, (S)-ibuprofen acid , isomaltooligosaccharides, 3-sialyllactose, *etc.*



Substrates

Biomacromolecule

or

Small molecule organic pollutants

or

Small molecule compound

Adsorption/Entrapment/
Covalent link/Cross-linking

1. Occlusion within a cross linked gel:-

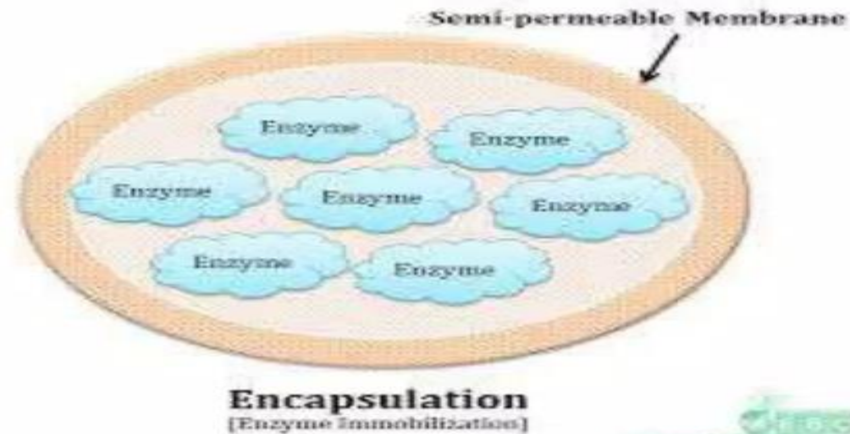
In this entrapment method, a highly cross-linked gel is formed as a result of the polymerization which has a fine "wire mesh" structure and can more effectively hold smaller enzymes in its cages.

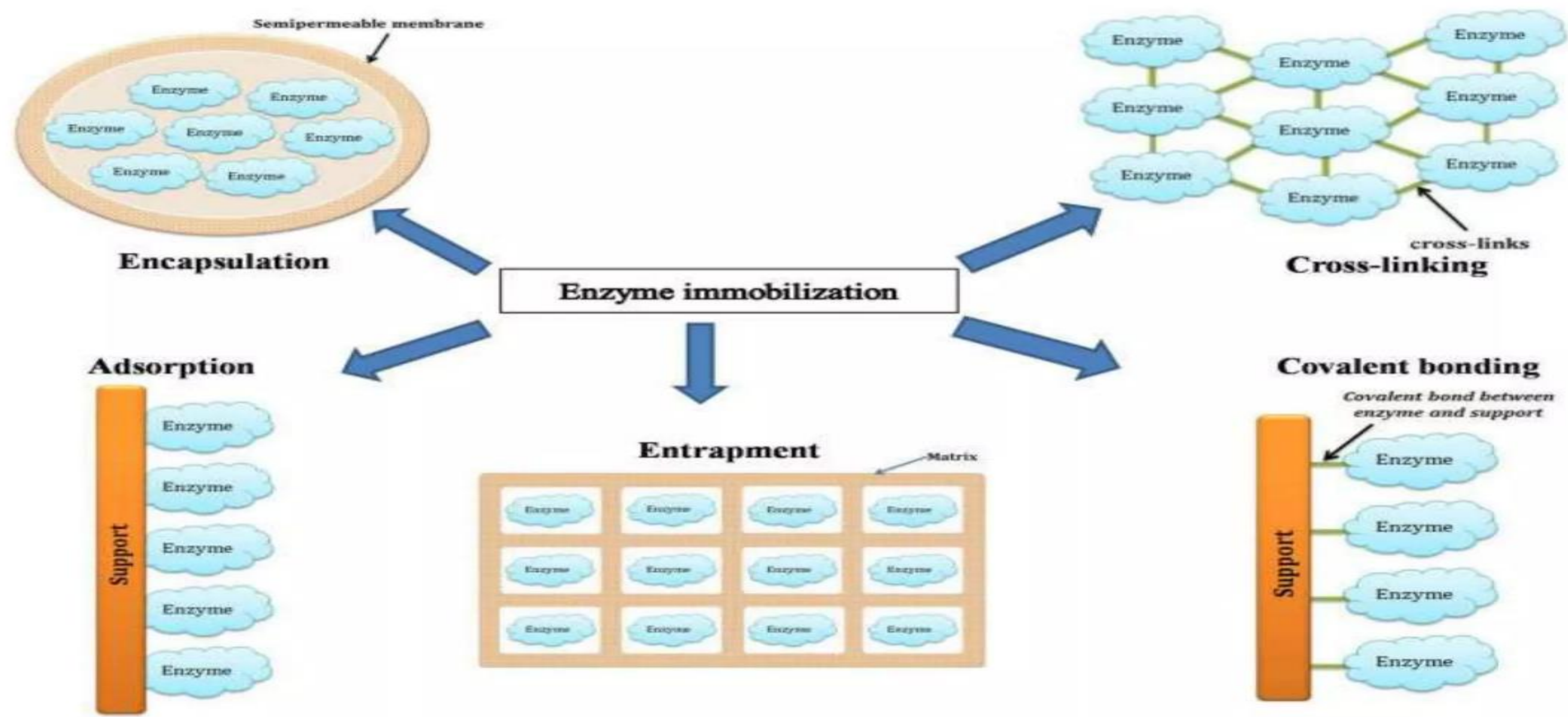
Amounts in excess of 1 g of enzyme per gram of gel or fibre may be entrapped.

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

2. Microencapsulation:-

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





Semipermeable membrane
Enzyme
Enzyme
Enzyme
Enzyme
Enzyme
Enzyme
Enzyme
Encapsulation
Enzyme
Enzyme
Enzyme
Enzyme
Enzyme
Enzyme
Enzyme
Enzyme
Enzyme
cross-links
Cross-linking
Enzyme immobilization
Adsorption
Support
Enzyme
Enzyme
Enzyme
Enzyme
Enzyme
Entrapment
Matrix
Covalent bonding
Covalent bond between enzyme and support
Support
Enzyme
Enzyme
Enzyme
Enzyme

Applications of Enzyme Immobilization

Enzyme	Method of Immobilization	Applications
Glucose isomerase	Embedment in collagen matrix concentration	Isomerization of glucose to fructose in production of high fructose syrup which is sweeter
Aminoacylase	Embedment in collagen matrix concentration	Resolution of racemic mixture of aminoacids to give L-aminoacids which have nutritional value
Glucose oxidase	Entrapment in polyacrylamide gel and layered over O_2 electrode	Determination of glucose concentration

Applications of Enzyme Immobilization

Enzyme	Method of Immobilization	Applications
Urease	Layered on Platinum/O2 electrode	Determination of urea and uric acid concentration
Alcohol oxidase	Layered on Platinum/O_2 electrode	Assay of alcohols
Alcohol phosphatase	Layered on Platinum/O_2 electrode	Assay of glucose-6-phosphate
Penicillinase	Layered on P^H electrode	Assay of penicillin

Applications of Enzyme Immobilization

Enzyme	Method of Immobilization	Applications
Urokinase/ Streptokinase	Adsorbed on Sephadex	Breakdown of thromboemboli
α-Amylase, catalase, trypsin	Adsorbed on dextran	Have slower clearance from blood
Phenylalanine ammonia	Encapsulation in microencapsules, fibres or gels	Reduction of blood levels of phenylalanine
Urease + adsorbed resin or charcoal	microencapsulation	Compact artificial kidney

Working and applications of Biosensors in pharmaceutical industries

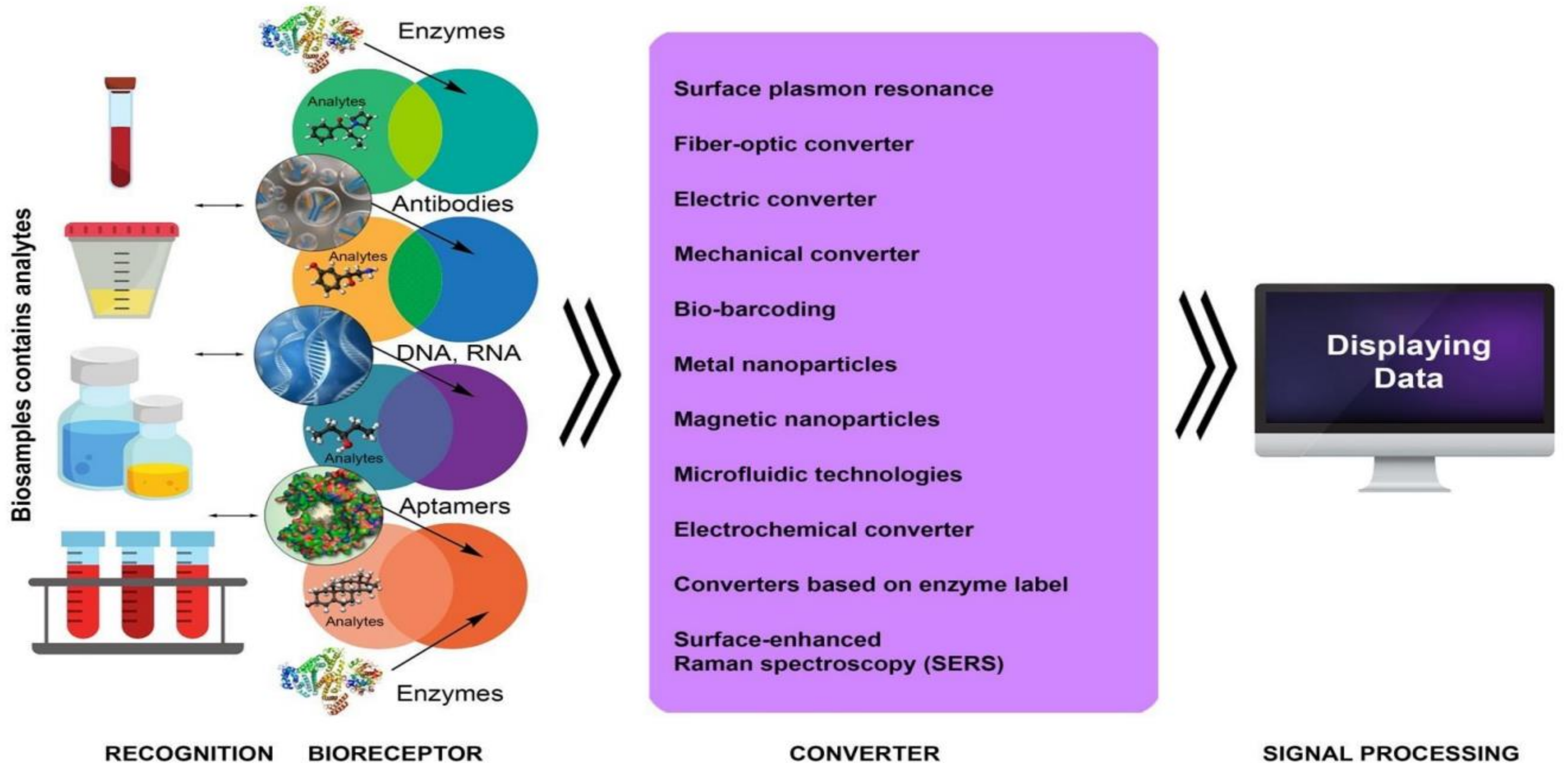
- BIOSENSORS: DEFINITION
- a) A biosensor is an analytical device which combines a biological component to detect an analyte and a physiological component to produce a signal which is measurable.
- b) The term Biological component involves any biological element like enzymes, tissues, cells, nucleic acids, antibodies, microorganisms etc.

B. COMPONENTS OF BIOSENSORS

- Different components of a typical biosensor are:
- a) **Analyte** A substance of interest needs detection. Example: Glucose is an 'analyte' in a biosensor designed to detect glucose.
- b) **Bioreceptor** A molecule that recognises the analyte specifically. A process of signal generation (in the form of light, heat, pH, charge or mass change etc.) upon interaction of the Bioreceptor with the analyte is termed as bio-recognition.
- c) **Transducer** Converts one form of energy into another. Convert the bio-recognition event into the measurable signal. The process of energy conversation is known as signalisation

- d) **Electronics** Part of biosensor that processes the transduced signal and prepares it for display. It is a complex electronic circuitry that performs steps such as amplification and conversion of signals from analogue into the digital form.
- Processed signals are then quantified by the display unit of the biosensor.
- e) **Display** Consists of a user interpretation system such as the liquid crystal display of a computer or a direct printer that generates number of curves. Output signal on the display can be numeric, graphic, tabular or an image, depending on the requirement of end user.

BIOSENSORS



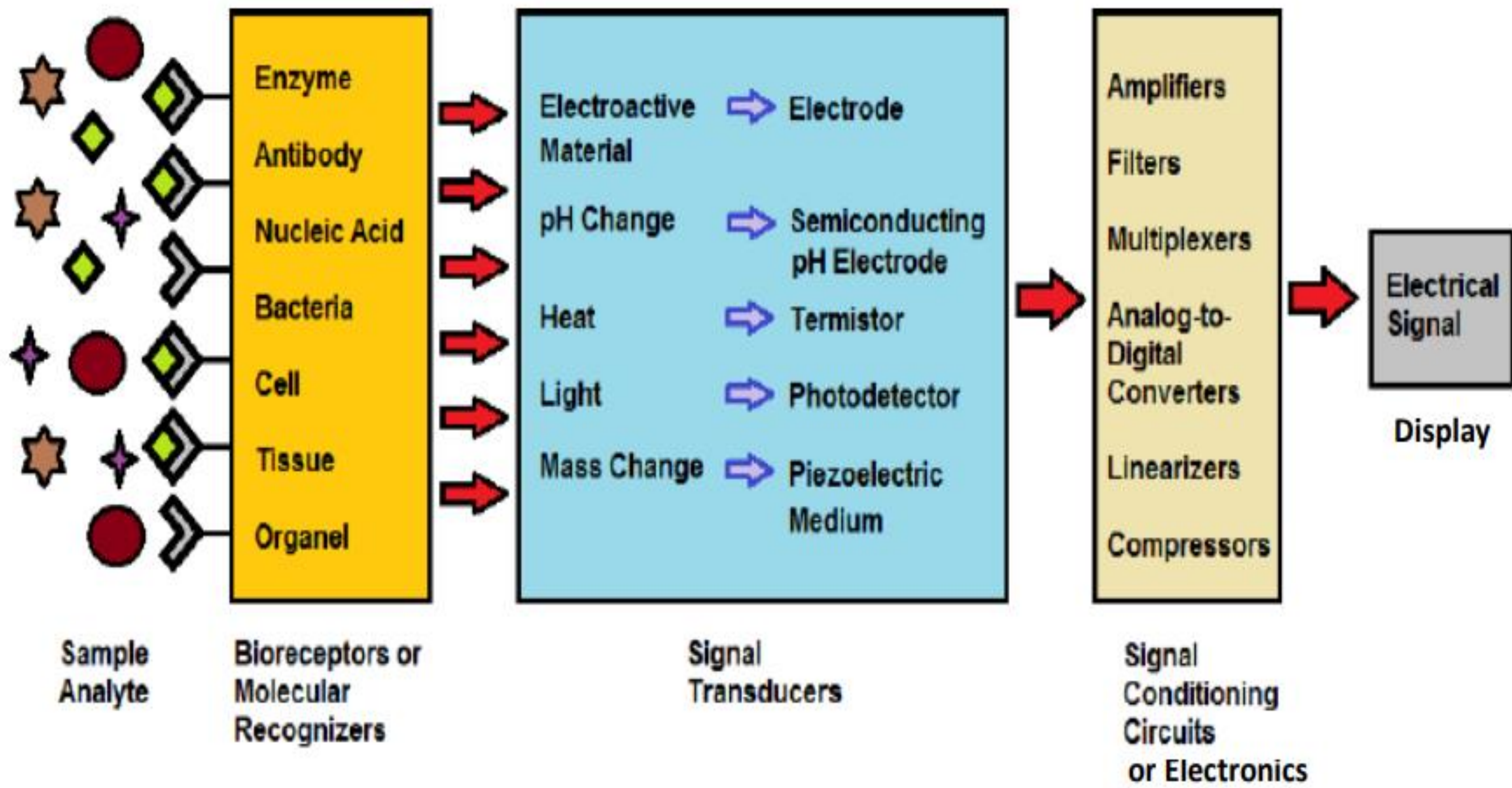


Figure 1: Components of Biosensors

C. CHARACTERISTICS OF BIOSENSORS

- a) Linearity- Maximum sensor output signal that can be detected by a sensor (should be high)
- b) Sensitivity- Magnitude of electrode response per unit substrate concentration
- c) Selectivity -Electrode response in the presence of other interfering chemicals or foreign materials (should be minimum)
- d) Stability- Maximum electrode response over a period of time
- e) Limit of detection- Lowest quantity of substance that can be determined from the absence of that substance (blank signal)

D. WORKING OF BIOSENSORS

- Any biosensor is functionally composed of three main components: Biological element, Transducer, and Detector.
- The biological element, essentially a Bioreceptor (enzyme, DNA, cell, antibody), is immobilized by conventional methods (physical or membrane entrapment, non-covalent or covalent binding).
- This immobilized biological material is in intimate contact with the transducer and allowed to interact with a specific analyte.
- The transducer measures heat, gas(O2), electrons or hydrogen as the result of interaction and outputs a signal.
- . The intensity of the signal output is proportional to the concentration of the analyte.
- The signal is then amplified and processed by the electron system

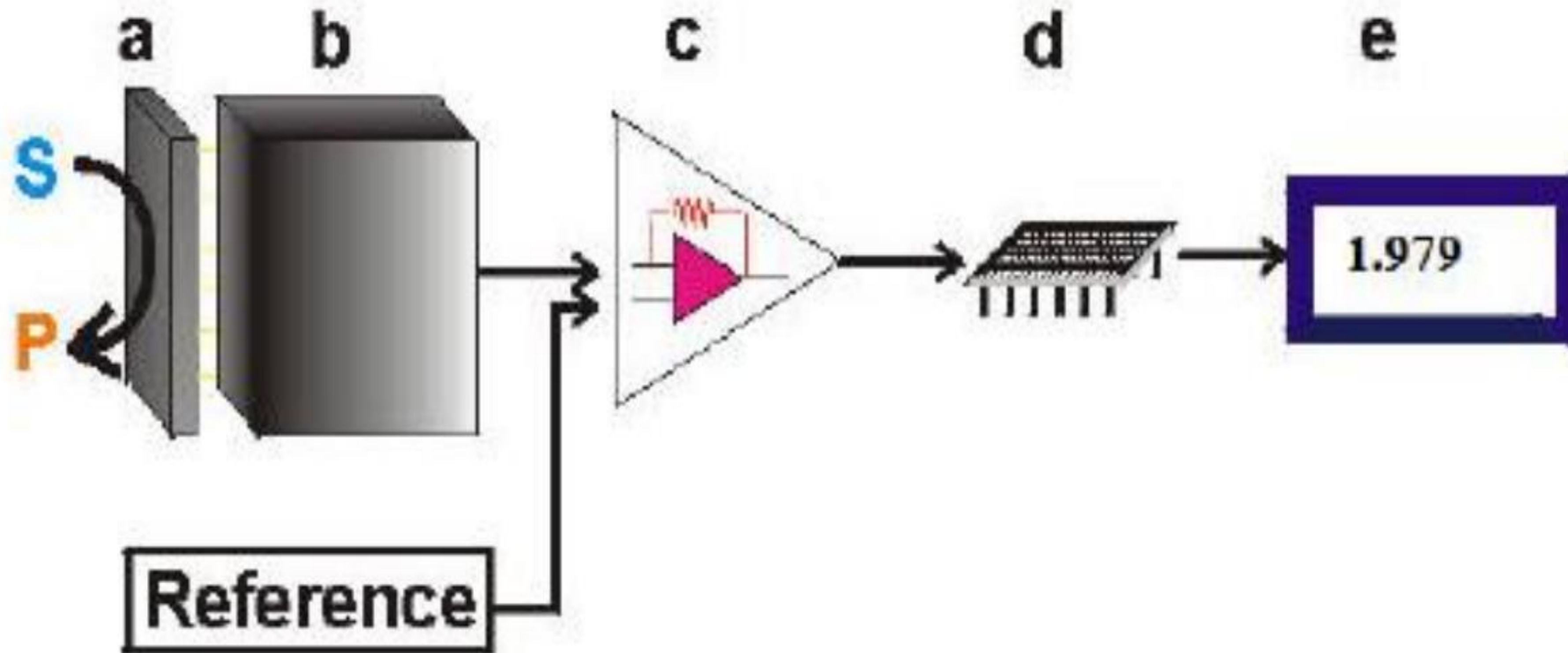


Figure 2: Functional components of a biosensor

a- Bioreceptor b-Transducer c-Amplifier d-Processor
e-Display S-Substrate P-Product

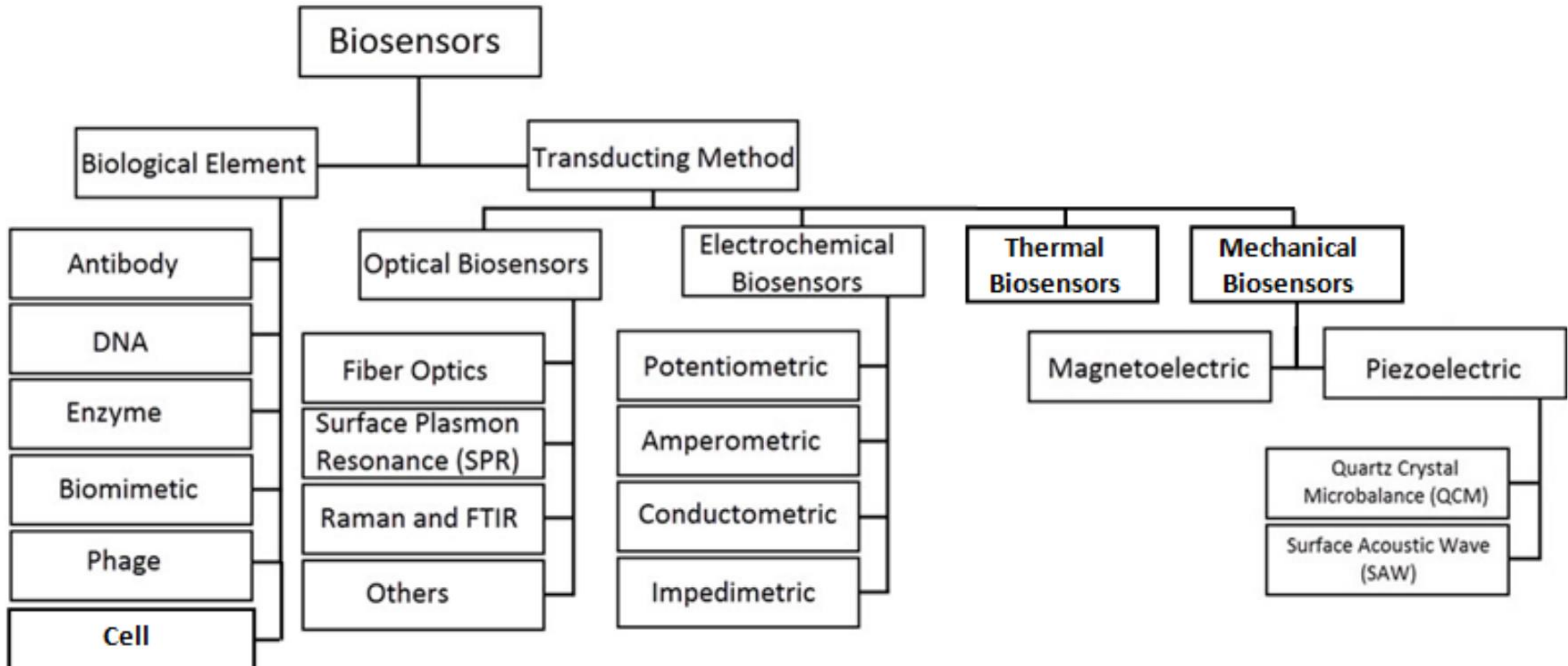
E. ADVANTAGES OF BIOSENSORS

- Rapid and continuous measurement
- High specificity
- Very little usage of reagents required for calibration
- Fast response time (typically less than a minute)
- Biosensors can easily detect analytes in the micromolar to nanomolar range.
- Ability to measure non-polar molecules that cannot be estimated by other conventional devices

F. DISADVANTAGES OF BIOSENSORS

- Heat sterilization is not possible as this would denature the biological part of the biosensor.
- The membrane that separates the reactor media from the immobilized cells of the sensor can become fouled by deposits.
- The cells in the biosensor can become intoxicated by other molecules that are capable of diffusing through the membrane.
- Changes in the reactor broth (i.e., pH) can put chemical and mechanical stress on the biosensor that might eventually impair it.
- **Biosensors can be classified** according to many criteria, out of which we mention the followings:
- a) The bioactive/bioreceptor material or bioelement
- b) The transducer

Figure 3: Various types of Biosensors



TYPES OF BIOSENSORS

- Electrochemical biosensor
- Optical biosensor
- Thermal biosensor
- Resonant biosensor
- Ion-sensitive biosensor

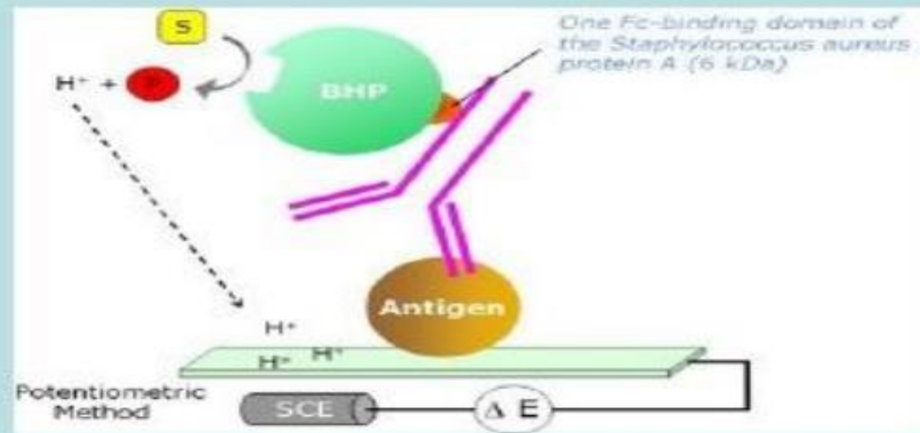
ELECTROCHEMICAL BIOSENSOR

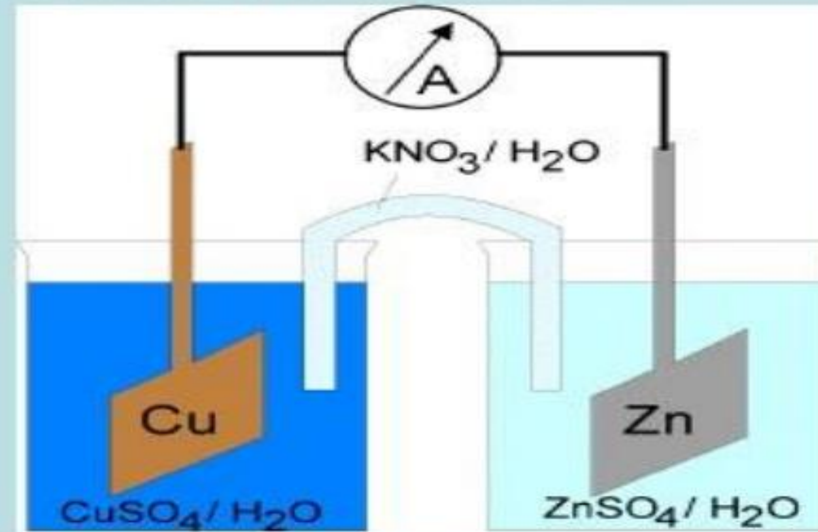
Principle:

- Many chemical reactions produce or consume ions or electrons which in turn cause some change in the electrical properties of the solution which can be sensed out and used as measuring parameter.
- **Classification:**
- 1. Amperometric Biosensors
- 2. Conductimetric Biosensors
- 3. Potentiometric Biosensors

Electrochemical biosensor

Electrochemical





Amperometric Biosensors

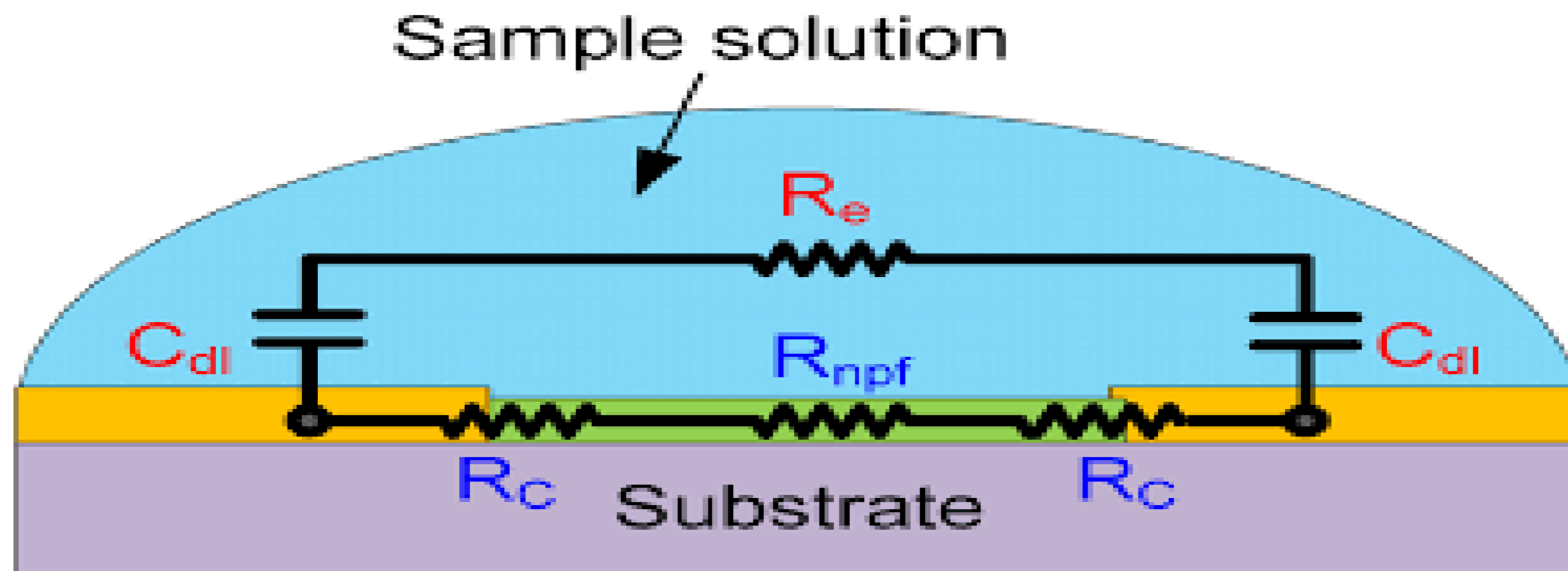
- The high sensitivity biosensor can detect electroactive species present in biological test samples.
- • Since the biological test samples may not be intrinsically electro-active, enzymes are needed to catalyze the production of radio-active species.
- • In this case, the measured parameters is current.
- • Translate a chemical event to an electrical event by measuring current passed (amperometric = most common), potential change between electrodes, etc.
- Oxidation reaction of the reduced chemical species
- Cred: Cred → Cox + ne

CONDUCTIMETRIC BIOSENSORS

- The measured parameter is the electrical conductance resistance of the solution.
- When electrochemical reactions produce ions or electrons, the overall conductivity or resistivity of the solution changes. This change is measured and calibrated to a proper scale (Conductance measurements have relatively low sensitivity).
- The electric field is generated using a sinusoidal voltage (AC) which in minimizing undesirable effects such as double layer charging and concentration polarization

CONDUCTIMETRIC BIOSENSOR

CONDUCTIMETRIC BIOSENSOR



POTENTIOMETRIC BIOSENSORS

- In this type of sensor the measured parameter is oxidation or reduction potential of an electrochemical reaction.
- The working principle relies on the fact that where a ramp voltage is applied to an electrode in solution, a current flow occurs because of electrochemical reactions.
- The voltage at which these reaction occurs indicate a particular reaction and particular species

POTENTIOMETRIC BIOSENSOR

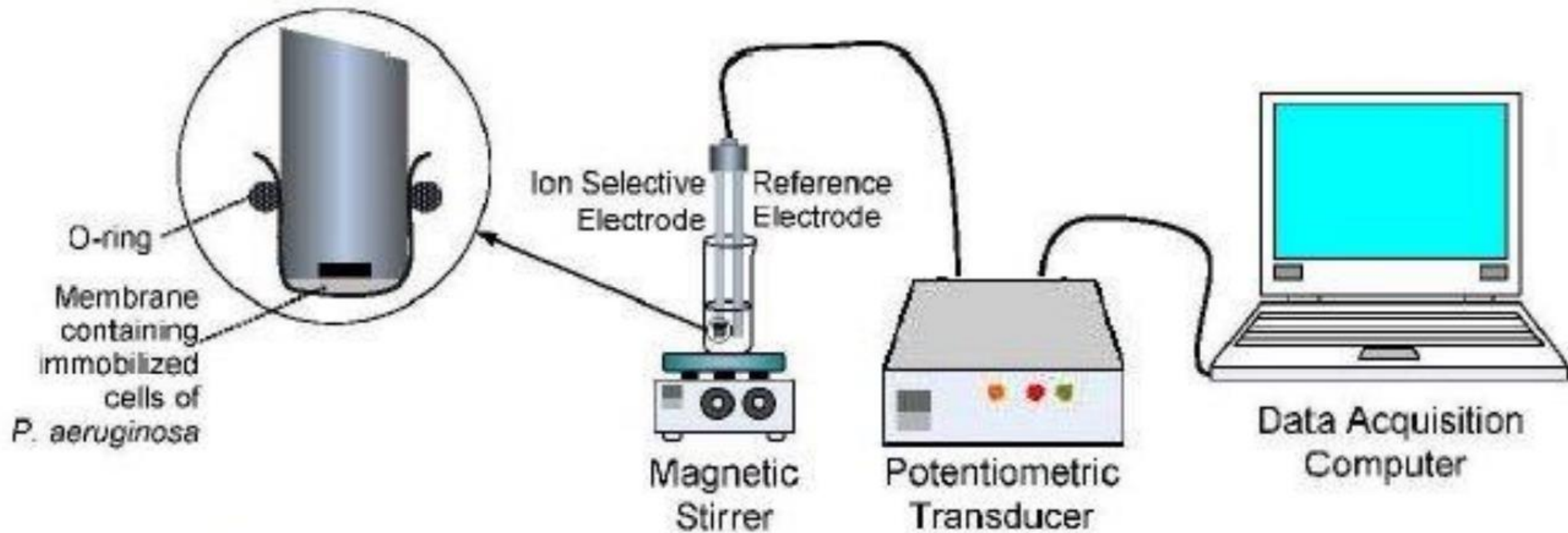
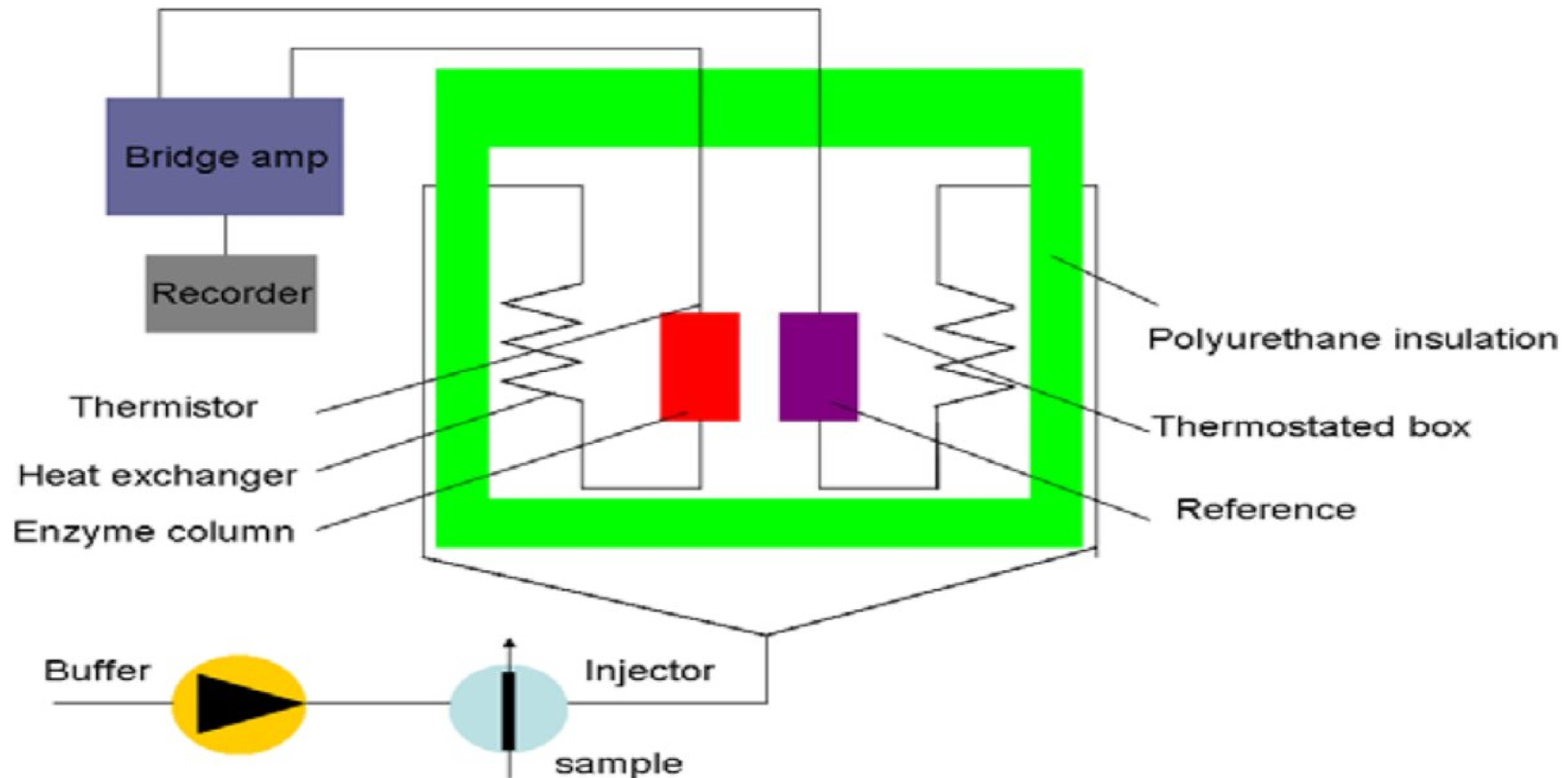


Figure 2. Experimental setup for potentiometric biosensor assays.

THERMAL DETECTION BIOSENSORS

- This type of biosensor work on the fundamental properties of biological reactions, namely absorption or production of heat , which in turn changes the temperature of the medium in which the reaction takes place.
- They are constructed by combining immobilized enzymes molecules with the temperature sensors. When the analyte comes in contact with the enzyme is measured and is calibrated against the analyte concentration.
- The total heat produced or absorbed is proportional to the molar enthalpy and the total number of molecules in the reaction.

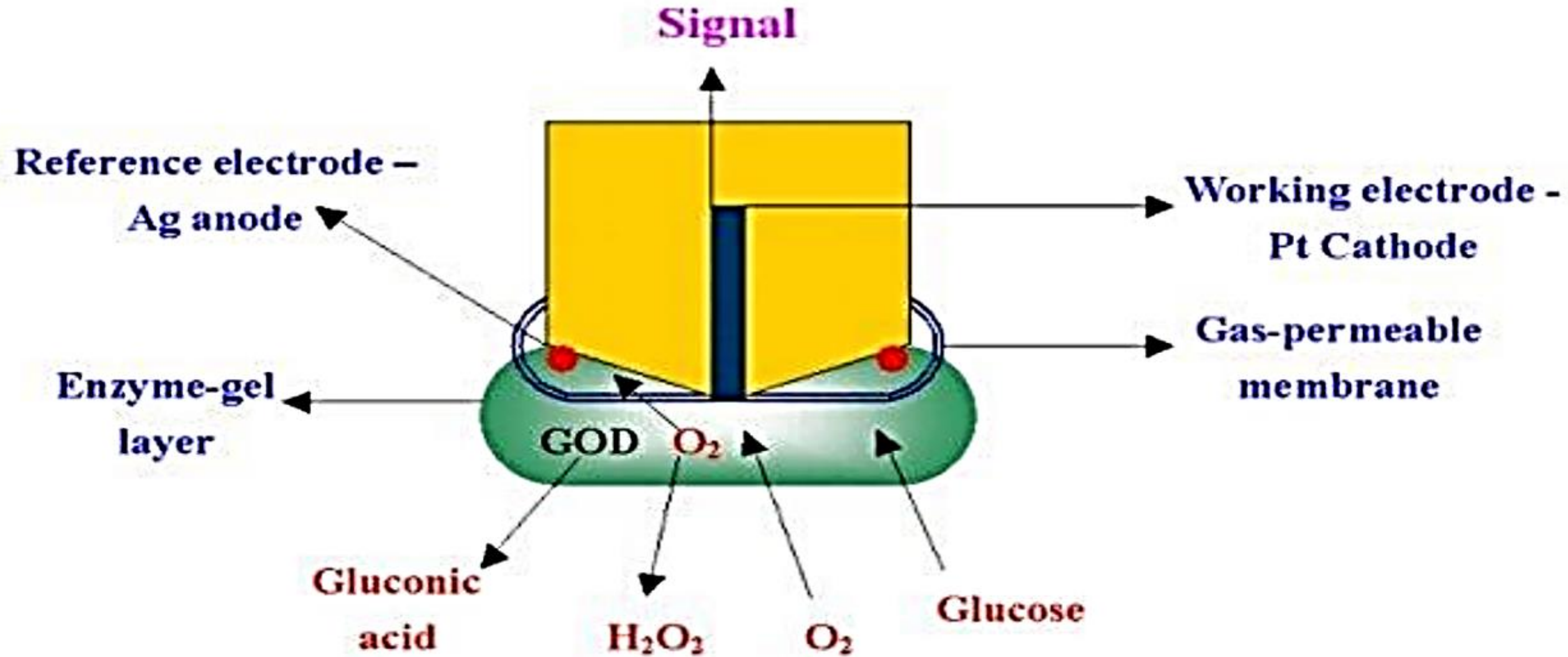
- Common applications of this type of biosensors includes the detection of pesticides and pathogenic bacteria.



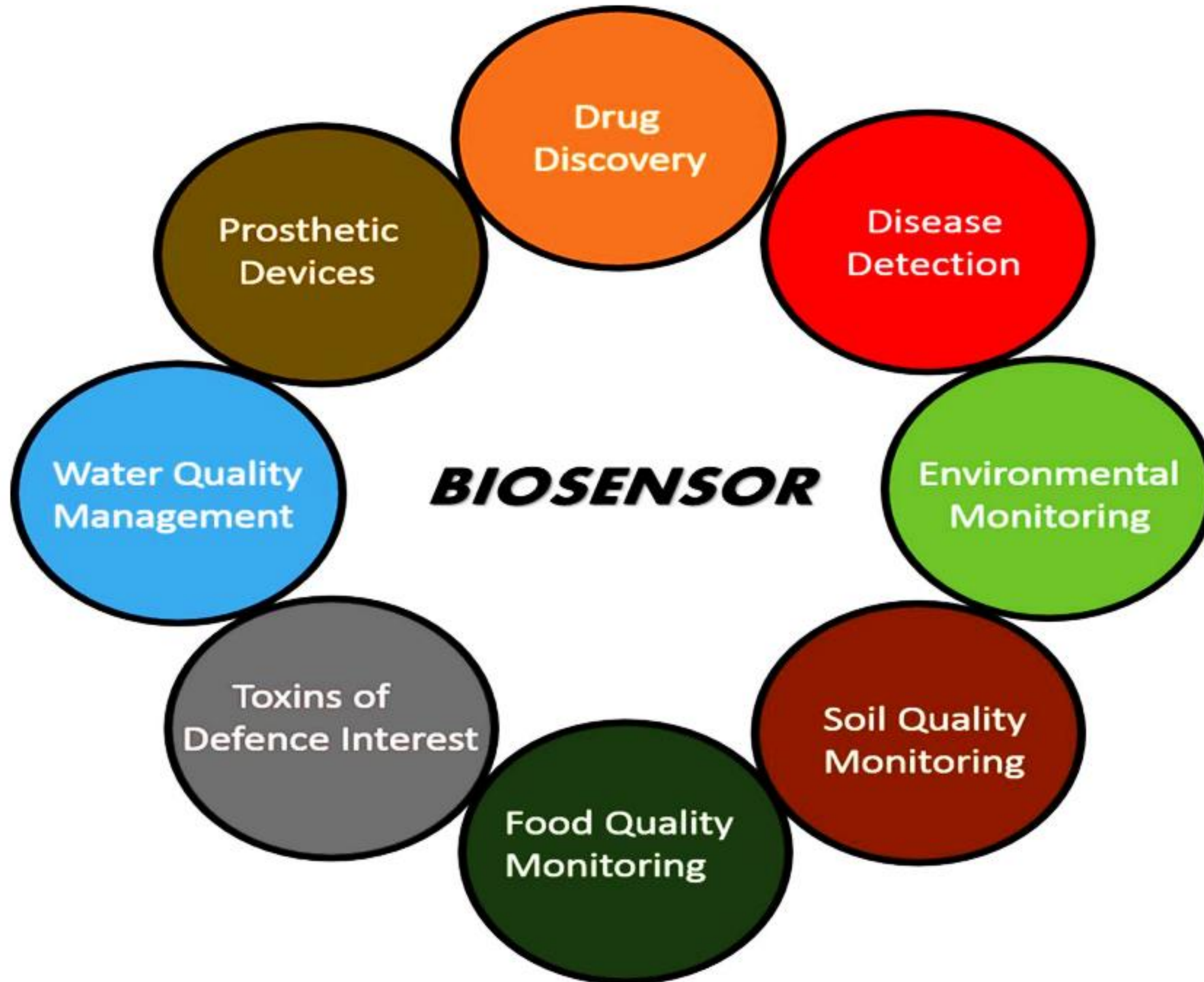
GLUCOSE BIOSENSORS

- Glucose reacts with glucose oxidase to form gluconic acid. Two electrons and two protons are also produced.
- Glucose mediator reacts with surrounding oxygen to form H2O2 and glucose oxidase.
- Now this glucose oxidase react with more glucose.
- Higher the glucose content, the higher the oxygen consumption.
- Glucose content can be detected by Pt-electrode

Glucose biosensors



Applications



- **CONCLUSION**
- As the potential threat to bioterrorism increase, there is great need for a tool that can quickly, reliably and accurately detect contaminating bio-agents in the atmosphere.

• Biosensors can essentially serve as low-cost and highly efficient devices for this purpose in addition to being used in other day-to-day application.

• Biosensors are known as immuno-sensors,optrodes, chemical, canaries, resonant mirrors, glucometers biochips bio-computers and so on

Protein engineering

- **A. DEFINITION**
- Modification of protein structure with recombinant technology or chemical treatment to get a desirable function for better use in medicine, industry and agriculture.
- It enables scientists to create unique materials that do not occur in nature.
- Protein engineering consists of three major approaches:
- **1) Rational design involves knowledge based mutagenesis (KBM) or site directed mutagenesis**
- **2) Computational protein design (CPD) or De Novo approach**
- **3) Directed evolution (DE) or irrational design or random mutagenesis**

B. APPROACHES OF PROTEIN ENGINEERING

- Protein engineering consists of three major approaches:
- **1) Rational design involves knowledge based mutagenesis (KBM)or site directed mutagenesis**
- Scientists use detailed knowledge of the structure and function of the protein to make desired changes
- **Advantages**
- Technically easy: Allows scientists to change structure of a protein in a predictable way.
- Relatively inexpensive

Disadvantages

- Detailed structural knowledge of the protein is often unavailable.
- It is extremely difficult to predict the effects of various mutations

2) Computational protein design (CPD) or De Novo approach

- Proteins are computationally designed from the level of amino acids to the level of a functional protein complex.
- This approach uses molecular modelling programs to predict amino acid sequences that will fold into a desired structure.
- Design scheme may encompass small regions of the proteins or the entire protein.
- Design may aim at the side chains or at the full backbone confirmation.

Advantages

- It has unique ability to design function de novo.
- It creates proteins with functions that are not available in naturally occurring proteins.
- Allows designing proteins with no human intervention in sequence selection.
- **Disadvantages**
- Tedious and complex process.

3) Directed evolution (DE) or irrational design or random mutagenesis

- Mimics the process of natural selection to steer (guide) proteins or nucleic acids towards a user-defined goal.
- Introduce desired properties into proteins via random mutation or gene recombination.
- **Advantages**:
- Can be performed without knowing every detail of a protein's structure.
- Library size limitation can be overcome by creating libraries of variants processing desired properties.
- **Disadvantages**:
- Time consuming and expensive process.

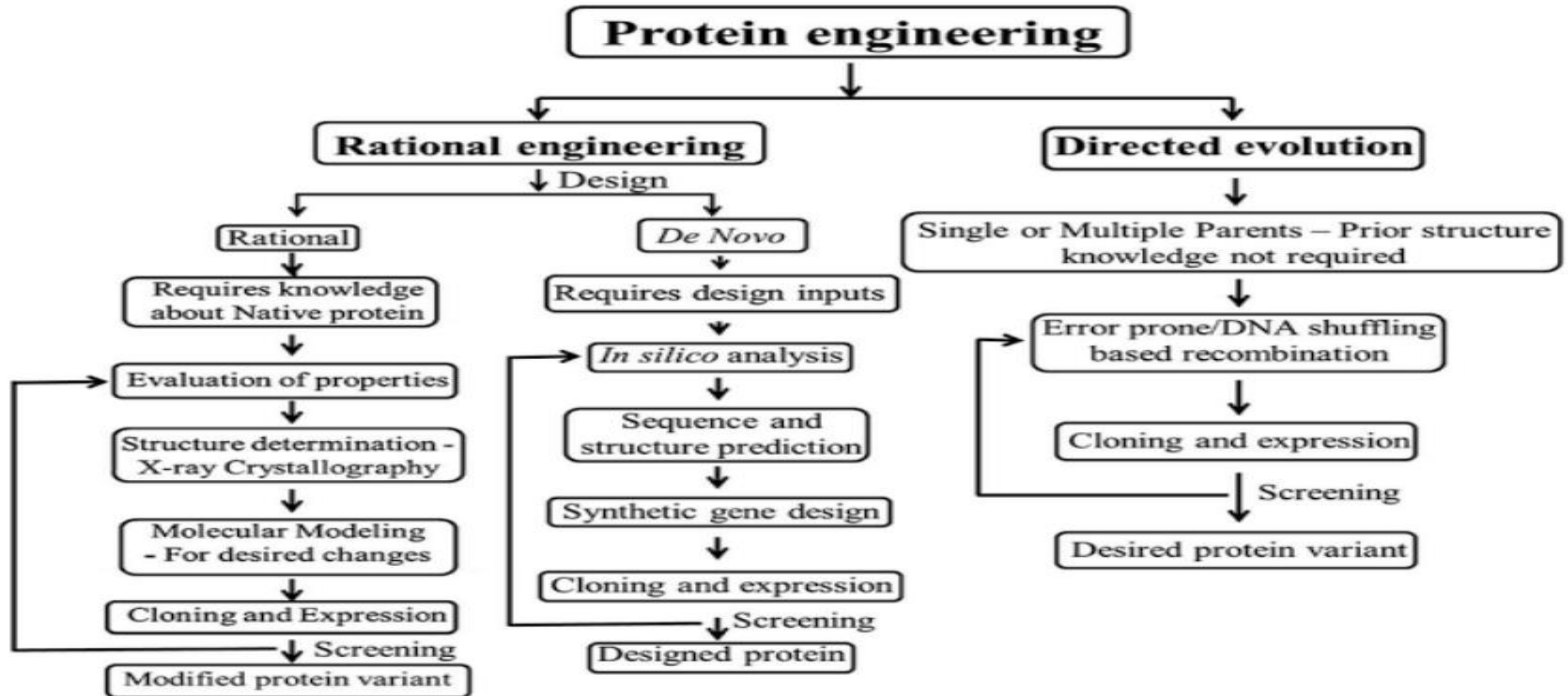


Figure 1: Different approaches of protein engineering

APPLICATIONS OF PROTEIN ENGINEERING

- **1) Industrial Applications**
- **Food Industry:**
- Proteases, Amylases, Lipases:
- **Detergent Industry**:
- Proteases: For removing protein stains
- Amylases: In removal of starch stains
- Lipases: In removal of lipid stains
- **2) Environmental Applications**
- Detoxification of inorganic pollutants (phenols, azo dyes, organophosphorous pesticides etc.) using enzymatic oxidation.
- In petrol biorefining:

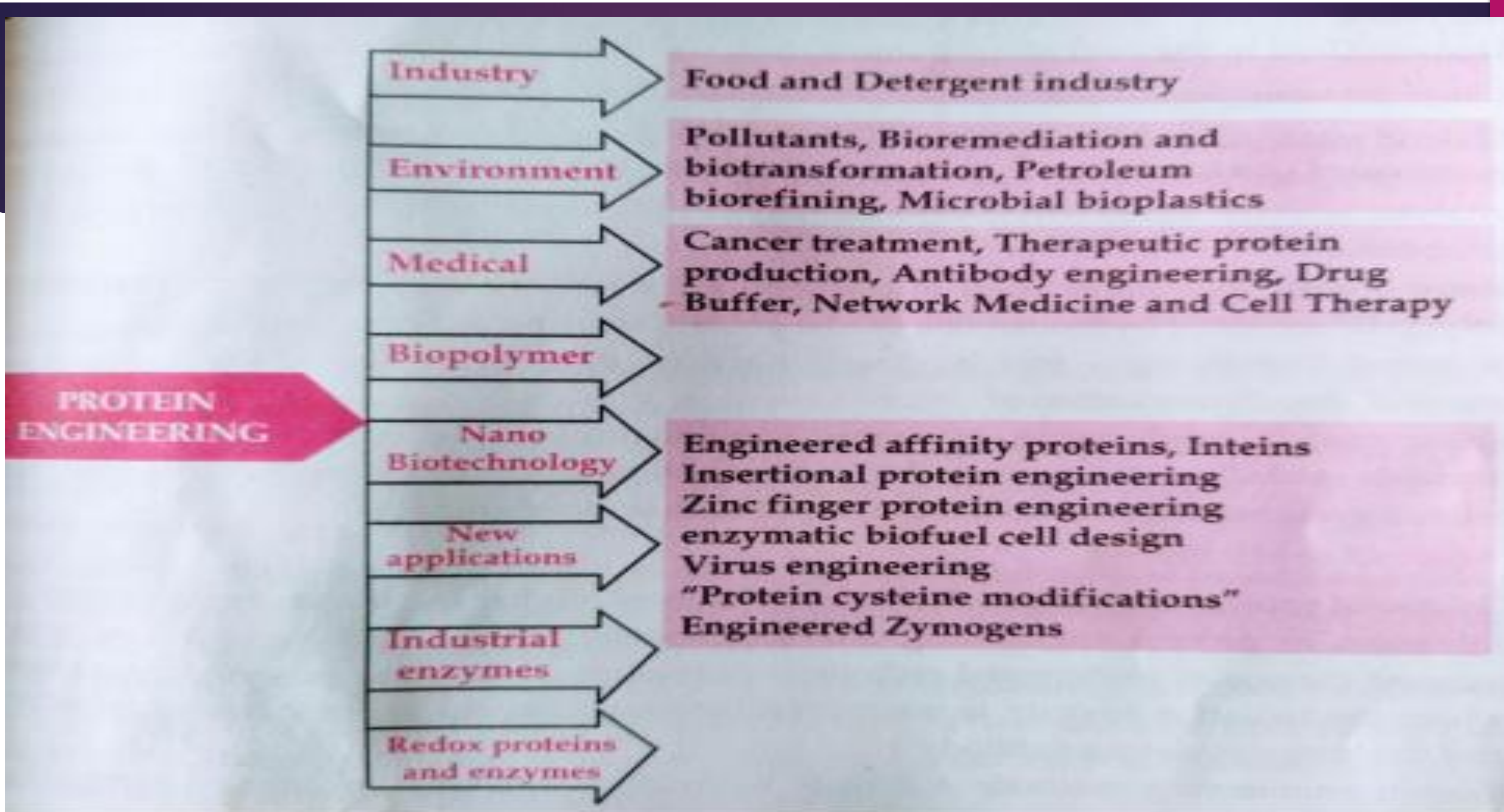


Figure 2: Various applications of protein engineering

- **Medical Applications**
- In pretargeted radio immunotherapy
- To improve pharmacokinetic properties of antibodies
- To modify antibodies to target cancer cells for clinical application

Uses of microbes in industry

USE OF MICROBES IN INDUSTRY. PRODUCTION OF ENZYMES–General consideration–Amylase, Catalase, Peroxidase, Lipase, Protease, Penicillinase

INDUSTRIAL USES

- Uses micro-organisms, typically grown on a large scale, to produce products or carry out chemical transformation.
- Originated with alcoholic fermentation process.
- Later on, used for the production of pharmaceuticals, food additives, enzymes and chemicals
- Major organisms used are fungi and bacteria (such as *Streptomyces*)

PROPERTIES OF USEFUL INDUSTRIAL MICROBES

- Produces spores or can be easily inoculated
- Should not be pathogenic (i.e. it shouldn't cause any disease
- Produces desired product quickly
- Grows rapidly on a large scale in inexpensive method

INDUSTRIAL PRODUCTS

- Beverages
- Antibiotics
- Organic acids
- Amino acids
- Enzymes
- Vitamins
- Organic solvents
- Steroids
- Vaccines
- Pharmaceutical drugs
- Dairy products

ANTIBIOTIC	PRODUCER ORGANISM
PENICILLIN	*Penicillium chrysogenum*
CEPHALOSPORIN	*Cephalosporium acremonium*
GRISEOFULVIN	*Penicillium griseofulvum*
BACITRACIN	*Bacillus subtilis*
POLYMYXIN B	*Bacillus polymyxa*
AMPHOTERICIN B	*Streptomyces nodosus*
ERYTHROMYCIN	*Streptomyces erythreus*
NEOMYCIN	*Streptomyces fradiae*
STREPTOMYCIN	*Streptomyces griseus*
TETRACYCLINE	*Streptomyces rimosus*
VANCOMYCIN	*Streptomyces orientalis*
GENTAMICIN	*Micromonospora purpurea*
RIFAMYCIN	*Streptomyces mediterranei*

(5)Enzymes

- Many microbes synthesize and excrete large quantities of enzymes into the surrounding medium. Using this feature of these tiny organisms, many enzymes have been produced commercially. These include **Amylase, Cellulase, Protease, Lipase, Pectinase, Streptokinase**, and many others.
- Enzymes are extensively used in **food processing and preservation, washing powders, leather industry, paper industry** and in scientific research.

ENZYMES	PRODUCER ORGANISM (Bacteria)
Penicillinase	*Bacillus cereus*
Amylase	*Bacillus coagulans*
L–asparaginase	*Citrobacter sp.*
Penicillin acylase	*Bacillus megaterium*
Protease	*Xanthomonas*

ENZYMES	PRODUCER ORGANISM (Fungi)
Amylase	*Aspergillus niger*
Lipase	*Candida lipolytica*
Invertase	*Saccharomyces cerevisiae*
Glucose oxidase	*Penicillium notatum*
Cellulase	*Trichoderma reesei*
Dextranase	*Penicillium funiculosum*
Trysinase	*Neurospora crassa*

PRODUCTION OF ENZYMES–General consideration–Amylase, Catalase, Peroxidase, Lipase, Protease, Penicillinase

- In early days, animal and plant sources largely contributed to production of enzymes.
- Even now, they act as the major source for certain enzymes
- Animal organs and tissues are very good sources for enzymes such as lipases, esterases and proteases.
- Some plants are excellent sources for certain enzymes–papain (papaya), bromelain (pineapple)

Cont..

- The most important limitations are the difficulty in isolating, purifying the enzymes and the cost factor.
- For this very reason, microbial production of enzymes is preferred.
- Microbes are the most significant and convenient sources of commercial enzymes
- They can be made to produce abundant quantities of enzymes under suitable growth conditions.
- Microbes can be cultivated by using inexpensive media and production can take place in a short period.

- Its easy to select microbes for the production of specific type of desired enzymes.
- Recovery, isolation and purification processes are easy with microbial enzymes than that with animal or plant sources.
- In fact, most enzymes of industrial applications have been successfully produced by microbes. Various fungi, yeast and bacteria are employed for this purpose.

Procedure for culturing of *Bacillus sp*. For inoculum preparation:-

Slant culture:-Inoculate *Bacillus sp* onto the surface of an agar culture and incubate it for 10-12 hr at 37°C. Store the slant culture at 4°C. Afterwards, regularly transfer Bacillus sp. to a new slant culture to maintain to bacteria strain.

Liquid seed culture:-Inoculate Bacillus sp. grown on the slant culture tin 50ml of liquid seed medium in a 250ml culture flask. Culture the bacteria at 37°C for 12-14hrs with a rotation speed at 200 rpm to make a liquid seed culture.

Basic flask culture:- add 6% above liquid seed culture (v/v) to 80ml basic fermentation culture in a 500ml culture flask and incubate at 37 °C, 200 rpm for 36-40 hrs.

Production methods

- Submerged fermentation (SMF)
- Solid state fermentation (SSF)

SUBMERGED FERMENTATION:-

1. It employs free flowing liquid substrates, such as molasses and broth
2. The products yielded in the fermentation are secreted into the fermentation broth.
3. This method is suitable for those microbes such as bacteria that require high moisture content for their growth.
4. The sterilization of the medium and purification process of the end products can be done easily.
5. Also the control of process parameters like temperature, pH, aeration, oxygen transfer and moisture can be done conveniently.

- **SOLID STATE FERMENTATION**:-

1. Method used for microbes which require less moisture content for their growth
2. The solid substrates commonly used in this method are bran, bagasse and paper pulp
3. The main advantage is that, nutrient-rich waste materials can be easily recycled and used as substrates in this method.
4. It requires simple instruments over SMF, higher concentration of products with less effluent generation

(1)AMYLASE

- Amylases are important hydrolase enzymes. It usually degrades complex polysaccharide molecules such as starch into glucose.
- Starch $\xrightarrow{\text{Amylase}}$ Glucose
- Present in saliva of humans (for digestion)
- This enzyme randomly cleave internal glycosidic linkages in starch molecules
- Hydrolysis of starch with amylase results in formation of **dextrin** and then disaccharide **maltose** (2 glucose molecules) and finally **glucose**.
- Types of amylases:-**α-amylase, β-amylase, γ-amylase**
- The substrate that α-amylase acts upon is starch.
- Starch is composed of 2 polymers- amylose (20-25% of the starch) and amylopectin (75-80% of starch).
- **Amylose** is broken down to give **maltotriose** and **maltose** molecule
- **Amylopectin** is broken down to give **dextrin** and **glucose** molecule

Cont..

- Primary source of β–amylase are the seeds of higher plants and sweet potatoes.
- BACTERIAL SOURCE OF α–Amylase:– *Bacillus subtilis, B. stearothermophilus, B. licheniformis, B. amyloliquefaciens*
- Carbon source:– maltose, sucrose, glucose
- Nitrogen source;– **Inorganic nitrogen sources** such as ammonium sulphate, ammonium chloride, ammonium hydrogen phosphate, **Organic sources** such as soyabean meal, peptone, yeast extract

Cont..

- *Bacillus licheniformis* has been used industrially for the production of α-amylases.
- The fermentation of bacterial amylases is accomplished in submerged culture at neutral pH and at a temperature of 30–40°C.
- Cereal meal and starch rich medium are used along with an organic source of nitrogen.
- After 10–20 hours, formation of α-amylase starts and continues for another 100 hours
- pH must be below 6 during fermentation to prevent the denaturation of α-amylase.

STEPS INVOLVED IN AMYLASE PRODUCTION

- **SELECTION OF MICROORGANISM**:–*Aspergillus niger, A. oryzae* as they produce amylase and it secretes acid so it easily degrades complex raw material.
- **SELECTION OF RAW MATERIALS**:–fungi cannot grow on natural media, so we provide synthetic or artificial media.

CHEMICALS	QUANTITY USED
Corn starch	24g/L
KCl	0.2g/L
Na_2HPO_4	4.7g/L
$CaCl_2/CaCO_3$	1g/L
$MgCl_2.6H_2O$	0.2g/L

Cont..

- **FERMENTATION PROCESS**

Fermenter is filled with the raw material or fermentation medium

Inoculate mycelium of *A. niger*

Maintain temperature at 30°C–45°C

After 72 hours recovery process is carried out.

Cont..

- **RECOVERY PROCESS**

Filtrate contains amylase, synthetic media and mycelia of fungi

By filtration, remove mycelia

Amylase is separated from synthetic media by adding ammonium hydroxide which forms precipitate with amylase

By filtration collect the precipitate (amylase+ammonium hydroxide)

Precipitate is subjected to crystallization at 4°C

Amylase in form of crystals are separated and collected

Cont..

- FUNGAL SOURCE OF α–Amylase:–*Aspergillus niger, Aspergillus oryzae, A. kawachii*
- They differ from bacterial amylases by low pH 4–5.
- The production of fungal amylase is carried out in solid–substrate culture and sometimes in submerged culture with selected strains of Aspergillus.
- Fermentation medium used remains the same as in the case of bacterial α–Amylase but the concentration of glucose inhibits the formation of amylase, therefore the concentration of glucose is kept low.
- Fungal amylases– used in manufacture of baked products and production of maltose rich syrups

(2)CATALASE

- Enzyme that catalyzes the decomposition of **hydrogen peroxide** into water and molecular oxygen.
- $2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$
- It is used to remove residual peroxide in application where hydrogen peroxide is added e.g. pasteurization or bleaching.
- **Uses**:-as preservative in food stuffs, removal of H_2O_2 from beverages, as anti-corrosive agent, in food industry (removal of H_2O_2 from pasteurized milk and dairy effluents), removal of H_2O_2 from blood.

PRODUCTION OF CATALASE

- SELECTION OF MICROORGANISM
- FORMULATION OF MEDIUM
- PRODUCTION PROCESS
- RECOVERY AND PURIFICATION OF ENZYMES

- **SELECTION OF MICROORGANISM**

  Organism selected should produce the maximum quantities of desired enzyme in a short time while the amount of other metabolite produced are minimum.

1. BACTERIA USED:–*Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus sp*.
2. FUNGI USED:–*Aspergillus fumigates, Candida albicans*

FORMULATION OF MEDIUM

1. Carbon source:-glucose, starch syrup, soluble starch may be used preferably
2. Nitrogen source:-Ammonium salt, nitrate salt, peptone, meat extract, yeast extract, corn steep liquor, soyabean powder
3. Minerals:-phosphates, magnesium salt, potassium salt, calcium salt, cobalt salt, zinc salt, molybdenum salts, copper salts

Composition of medium:-

Chemicals	Quantity
Glucose	10g
$NaNO_3$	5g
$MgSO_4.7H_2O$	0.5g
Na_2HPO_4	9.52g
KH_2PO_4	0.6g
$FeSO_4.7H_2O$	0.0026g
Distilled water	1L

*pH =7-7.5
*temperature=40-60°C

- **PRODUCTION PROCESS**

1. Production of catalase is done by submerged fermentation of *A. niger*
2. Fermenter is filled with the medium and sterilized first, then the pre-incubated broth of *A. niger* grown in the same medium is added as a seed in the fermentor by inoculating.
3. pH 7-7.5
4. The aerated and stirred fermentor has a total fermentation period 72 hours
5. Anti-foaming agents can be added to prevent froth formation

- **RECOVERY AND PURIFICATION OF ENZYMES**

The medium is filtered to remove the mycelia.

Concentrate the broth by ultrafiltration

The enzyme was subjected to lyophilization to get solid form of enzyme preparation

(3)PEROXIDASE

- Peroxidase is an enzyme that decomposes hydrogen peroxide in to water and molecular oxygen.
- $H_2O_2 \xrightarrow{\text{peroxidase}} H_2O + O_2$
- They are named after the fact that they commonly break up peroxides (toxic substances) and form non-toxic substance
- Hydrogen peroxide is a by-product of using oxygen for respiration.
- **USES:**-In decomposition of pollutants, paper industries, dye decolorization, sewage treatment, treatment of industrial waste water

- They are abundantly found in bacteria, fungi, algae, plants and animals.
- BACTERIAL source:–*Bacillus subtilis, Pseudomonas sp., Citrobacter sp.*
- FUNGAL source:–*Candida krusei, Coprinopsis cinerea*

PRODUCTION OF PEROXIDASE

- **Selection of microorganism:**–Pure culture of *Bacillus subtilis* or *Aspergillus niger* was selected. pH=6–7 and temperature=25°C.
- **Formulation of medium**
- **Production process**
- **Enzyme extraction and purification**

- **SELECTION OF MICROORGANISM**:–*Aspergillus niger* was maintained in Potato dextrose agar (PDA) media at 4°C. Inoculum was prepared by adding 10 ml of sterilized distilled water to 5 days old PDA slant culture. 5% of inoculum was added for production of enzyme.

- **FORMULATION OF MEDIUM:-**

CHEMICALS	QUANTITY (g/L)
Glucose	10
Yeast extract	2
NH_4NO_3	0.2
$Mg.SO_4.7H_2O$	0.5
K_2HPO_4	1
$NaH_2PO_4.H_2O$	0.4
Distilled water	1 L

- **PRODUCTION PROCESS:-**

1. By fermentation process, 5 ml of inoculum was added in 100 ml of production medium by using sterilized disposable syringe.
2. It was incubated for 12 days at pH 6.5 and temperature 25°C.

- **ENZYME EXTRACTION AND PURIFICATION:-**

1. Removal of debris by filtration
2. Add ammonium sulphate to precipitate the enzyme and pass it through ion exchange resins.

(4)LIPASE

- It is an enzyme that catalyzes the breakdown of most triglycerides into fatty acids and glycerol (lipolysis).
- Lipase enzyme is usually found naturally in pancreatic juice and stomach.
- They also control the volume of fat in body that is synthesized and burned by reduction of adipose tissue.
- Lipase can be purified or extracted from plant, animal, yeast, bacteria and fungal sources.

- **Uses**:-used in detergents (laundry) due to the wide use of washing machine,

  -fat and oil processing (inexpensive and less needed lipid can be converted into greater value fat), PUFAs (Poly Unsaturated Fatty Acids) used remarkably as pharmaceutical,

  -nutraceutical obtained by using microbial lipases from plant, and animal lipids.

PRODUCTION OF LIPASE

- Selection of microorganism
- Formulation of medium
- Production process
- Recovery and purification of enzymes

SELECTION OF MICROORGANISM

1. Bacterial source:-*Pseudomonas aeruginosa, Staphylococcus caseolyticus, Bacillus coagulans, Bacillus subtilis, Bacillus stearothermophilus*
2. Fungal source:-*Aspergillus niger, Candida rugosa, Candida utilis, Penicillium citrinum, Penicillium restrictum, Candida cylindracea*
3. *Candida cylindracea* was grown on yeast agar medium and maintained at 4°C. A loop full of cells from freshly grown culture (agar slant) of Candida cylindracea was transferred to flask. Flask was then incubated at 30°C on a rotary shaker at 200 rpm for 36 hrs.

- **FORMULATION OF MEDIUM**:–medium containing flask was sterilized at in an autoclave at 121°C for 20 min

CHEMICALS REQUIRED	QUANTITY (g/L)
KH_2PO_4	6
$MgSO_4.7H_2O$	1
Urea	4
$FeCl_3.6H_20$	10 mg
Inositol	0.4 mg
Thiamine hydochloride	0.2 mg
Biotin	0.8 mg
Glucose	10 mg
Distilled water	1L

PRODUCTION PROCESS:-

1. The medium used in preparing the inoculum is the same as the production medium. The only difference is the use of glucose as the carbon source.
2. Palm oil can be used as the main carbon source in production medium but not used in the inoculum preparation medium
3. 50g of sterile palm oil was transferred aseptically to the sterilized medium in the fermentor
4. 10% of the inoculum was added to the total volume.
5. Samples of 50ml was withdrawn aseptically at regular time interval for analysis.
6. Temperature=28-33°C, pH=6-7, stirring speed=500rpm

- RECOVERY AND PURIFICATION OF ENZYMES:–

1. Removal of debris by filtration
2. After fermentation the extract was precipitated with ammonium sulphate
3. Ultrafiltration was used to concentrate the sample

(5)PROTEASE

- These are a group of enzymes belonging to a class of hydrolases whose catalytic function is to hydrolyze peptide bonds of proteins
- They are also called proteolytic enzymes
- **USES**:–for cleaning contact lenses to remove large variety of stains of food, blood and body secretions

  –in the manufacture of cheese

  –meat tenderization (reduce toughness of meat)

  –to correct lytic enzyme deficiency syndrome

  –treatment of industrial waste:– keratinase used as depilatory to remove hair from drains

PRODUCTION OF BACTERIAL PROTEASES

- SELECTION OF MICROORGANISM
- FORMULATION OF MEDIUM
- PRODUCTION PROCESS
- RECOVERY AND PURIFICATION OF ENZYMES

- SELECTION OF MICROORGANISM

1. Bacterial source:- *Bacillus licheniformis, B. amyloliquefaciens, B. stearothermophilus*
2. High carbohydrate level in the medium stimulates protease production

FORMULATION OF MEDIUM

1. Media used contains ground barley as the carbon source
2. Starch level is limited
3. Protein hydrolysates or sodium glutamate is used as the nitrogen source

PRODUCTION PROCESS

1. The medium has a pH of 6.5–7.5 and incubation temperature of 37°C
2. Fermentation proceeds for 3–5 days

Preserved inoculum → Inoculum development → Inoculation tank → Fermenter → Cell disruption Filtration (to remove debris) → Remove nucleic acids → Salt treatment → Cool storage → Fitration → Final purification (chromatography etc.) → Freeze drying

*During fermentation, insoluble protein of the medium is partially hydrolysed by boiling in dilute acid/enzymatic treatment.

- **RECOVERY AND PURIFICATION OF ENZYMES**

1. Culture is filtered
2. Aqueous portion is concentrated by evaporation at reduced pressure and at temperature not less than 40°C
3. Recovery can also be carried out by precipitation.

PRODUCTION OF FUNGAL PROTEASE

- **SELECTION OF MICROORGANISM**

1. Various fungi produce protease such as *Aspergillus oryzae, A. sojae, A. niger, A. wentii, Mucor pusillus, Mucor miehei, Mucor delemar, Amylomyces rouxii*

FORMULATION OF MEDIUM

1. The fungus is grown on wheat bran under fermentation conditions similar to those for amylase production
2. Trace elements used in Inoculation medium:–

CHEMICALS REQUIRED	QUANTITY
$Fe(NH_4)_2(SO_4)_2$	1 mg
$ZnSO_4$	1 mg
$MnSO_4$	0.5 mg
$CuSO_4$	0.08 mg
$CoSO_4$	0.1 mg
H_3BO_3	0.1 mg
Distilled water	1 L

- Medium for inoculum preparation (g/L):-

Chemicals required	Quantity
Casein hydrolysate	5
Soya protease digest	2
Yeast extract	2
Soluble starch	10
D-mannitol	5
Trace element	1 ml
$FeSO_4.7H_2O$	15 mg

- Medium for production (g/L):-

Chemicals required	Quantity
Soyabean meal	30
Glucose	10
$NaNO_3$	3
Skim milk	10
KH_2PO_4	0.5
$MgSO4.7H_2O$	0.25

- **PRODUCTION PROCESS**

1. *Endothia parasitica* is employed for the production of this enzyme in lab scale
2. A 5% inoculum from 96 hrs flask is utilized for the production in a stirred, aerated vessel for 48 hrs at 28°C.
3. Optimum pH is 4–5
4. Proteases from *Aspergillus oryzae* and *A. sojae* are produced by solid substrate fermentation.

RECOVERY AND PURIFICATION OF ENZYMES

1. After growth the harvest can be dried at 50°C or less
2. Protease can also be extracted by water followed by addition of alcohol for precipitation and dried at 55°C

(6)PENICILLINASE

1. Specific type of β–lactamase enzymes which are produced by certain bacterias showing specificity for breaking β–lactam ring (by hydrolysing the ring) and responsible for their resistance of bacteria to β–lactam antibiotics like Penicillin and Carbapenems.
2. **USES**:–for treating penicillin induced allergy like serum sickness, acute urticaria

   – for measuring human prolactin in plasma

   –for cleaning the apparatus such as flow plates, contact plates used in production of antibiotics, to neutralize the anti–microbial activity.

PRODUCTION OF PENICILLINASE

- SELECTION OF MICROORGANISM
- FORMULATION OF MEDIUM
- PRODUCTION PROCESS
- RECOVERY AND PURIFICATION OF ENZYMES

- **SELECTION OF MICROORGANISM**

1. *Bacillus cereus* isolated from the soil sample can be used for the production of penicillinase.

FORMULATION OF MEDIUM

1. Culture media for growth of organism:–

Chemicals Required	Quantity (g/L)
Glucose or glycerol	2
Polypeptone	5
Yeast extract	5
K_2HPO_4	1
$MgSO_4$	0.2
Distilled water	1 L

2. Adjust the pH to 9 with 10% Sodium bicarbonate which was sterilized separately
3. Optimum temperature was adjusted from 30–37°C

- **PRODUCTION PROCESS**

1. Culture medium is inoculated with 19 hr old inoculum culture
2. Glucose or glycerol (0.2%) is added to enhance enzyme production
3. Cultivate at 30°C temperature and pH 9 for 23 hr on a rotary shaker.
4. Then add 10,000 units of Benzyl penicillin as an inducer of penicillinase and cultivation was further continued for 4 hr (to yield maximum enzyme)

RECOVERY AND PURIFICATION OF ENZYME

1. 5 day culture is centrifuged and the cell debris is discarded to separate the filtrate
2. 1 liter quantity of filtrate at pH 6.5 was stirred in the cold for 30 min with 50 g of Hyflo supercel (diatomaceous silica for filtration i.e. filter aid) and the adsorbing agent collected by filtration
3. Supercel was suspended in 500 ml of ammonia water with mechanical stirring and elution carried out in the cold (4°C) for 30 min.
4. Add ammonium sulphate to precipitate out the enzyme as tiny floccules, which is removed by filtration (by using glass filter)
5. Dry it using freeze dryer (lyophilization)
6. The final product obtained is grayish brown in colour and of a light, flaky or leathery consistency

- Thank You