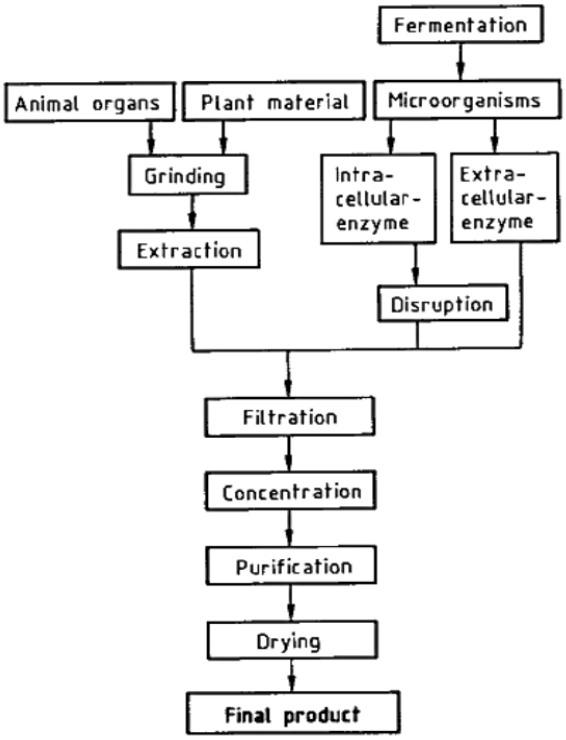
1. Production and purification of crude enzyme extracts from plant, animal and microbial sources

The degree of purity of commercial enzymes ranges from raw enzymes to highly- purified forms and depends on the application. Raw materials for the isolation of enzymes are animal organs, plant material, and microorganisms. Enzymes are universally present in living organisms; each cell synthesizes a large number of different enzymes to maintain its metabolic reactions. The choice of procedures for enzyme purification depends on their location. Isolation of intracellular enzymes often involves the separation of complex biological mixtures. On the other hand, extracellular enzymes are generally released into the medium with only a few other components. Enzymes are very complex proteins, and their high degree of specificity as catalysts is manifest only in their native state. The native conformation is attained under specific conditions of pH, temperature, and ionic strength.Hence, only mild and specific methods can be used for enzyme isolation.

Sequence of steps in the isolation of enzymes



1.1 Preparation of Biological Starting Materials

Animal Organs : Animal organs must be transported and stored at low temperature to retain enzymatic activity. The organs should be freed of fat and connective tissue before freezing. Frozen organs can be minced with machines generally used in the meat industry, and the enzymes can be extracted with a buffer solution. Besides mechanical grinding, enzymatic digestion can also be employed. Fat attached to the organs interferes with subsequent purification steps and can be removed with organic solvents. However, enzymatic activity might be influenced negatively by this procedure.

Plant Material : Plant material can be ground with various crushers or grinders, and the desired enzymes can be extracted with buffer solutions. The cells can also be disrupted by previous treatment with lytic enzymes.

Microorganisms : Microorganisms are a significant source of enzymes. New techniques, summarized under genetic and protein engineering, have much to offer the enzyme industry.A gene can be transferred into a microorganism to make that organism produce a protein it did not make naturally. Alternatively, modification of the genome of a microorganism can change the properties of proteins so that they may be isolated and purified more easily. Such modifications might, for example, cause the release of intracellular enzymes into the medium; change the net charge and, therefore, the chromatographic properties of proteins; or lead to the formation of fused proteins.

Most enzymes used commercially are extracellular enzymes, and the first step in their isolation is separation of the cells from the solution. For intracellular enzymes, which are being isolated today in increasing amounts, the first step

involves grinding to rupture the cells. A number of methods for the disruption of cells are known, corresponding to the different types of cells and the problems involved in isolating intracellular enzymes. However, only a few of these methods are used on an industrial scale.

Mechanical methods	Nonmechanical methods
High pressure (Manton–Gaulin, French-press)	Drying (freeze-drying, organic solvents)
Grinding (ball mill)	Lysis
Ultrasound	physical: freezing, osmotic shock
	chemical: detergents, antibiotics
	enzymatic: enzymes (e.g., lysozyme), antibiotics

1.1.1 Cell Disruption by Mechanical Methods

High-pressure homogenization is the most common method of cell disruption. The cell suspension is pressed through a valve and hits an impact ring (e.g., Manton–Gaulin homogenizer). The cells are ruptured by shearing forces and simultaneous decompression. Depending on the type of machine, its capacity ranges from 50 to 5000 L/h.
The rigid cell walls of small bacteria are only partially ruptured at the pressures up to 55 MPa (550 bar) achieved by this method. Higher pressures, however, would result in further heat exposure (2.2 8C per 10 MPa). Hence, the increased enzyme yield resulting from improved cell disruption could be counteracted by partial inactivation caused by heating and higher shearing forces. Therefore, efficient cooling must be provided.

The ***wet grinding*** of cells in a high-speed bead mill is another effective method of cell disruption [190–193]. Glass balls with a diameter of 0.2–1 mm are used to break the cells. The efficiency of this method depends on the geometry of the stirrer system. A symmetrical arrangement of circular disks gives better results than the normal asymmetrical arrangement. Given optimal parameters such as stirring rate, number and size of glass beads, flow rate, cell concentration, and temperature, a protein release of up to 90 % can be achieved in a single passage.

1.1.2 Cell Disruption by Nonmechanical Methods

Cells may frequently be disrupted by ***chemical, thermal, or enzymatic lysis***. The drying of microorganisms and the preparation of acetone powders are standard procedures in which the structure of the cell wall is altered to permit subsequent extraction of the cell contents.Methods based on enzymes or autolysis have been described in the literature. Ultrasound is generally used in the laboratory. In this procedure, cells are disrupted by shearing forces and cavitation. An optimal temperature must be maintained by cooling the cell suspension because heat is generated in the process.

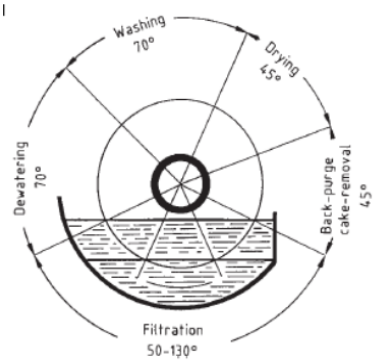
1.2 Separation of Solid Matter

After cell disruption, the next step is separation of extracellular or intracellular enzymes from cells or cellular fragments, respectively. This operation is rather difficult because of the small size of bacterial cells and the slight difference between the density of the cells and that of the fermentation medium. Continuous filtration is used in industry. Large cells, e.g., yeast cells, can be removed by decantation. Today, efficient centrifuges have been developed to separate cells and cellular fragments in a continuous process. Residual plant and organ matter can be separated with simpler centrifuges or filters.

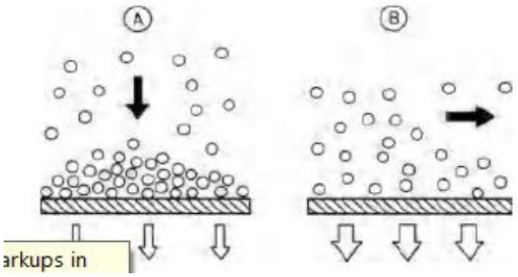
1.2.1 Filtration

The filtration rate is a function of filter area, pressure, viscosity, and resistance offered by the filter cake and medium. For a clean liquid, all these terms are constant which results in a constant flow rate for a constant pressure drop. The cumulative filtrate volume increases linearly with time. During the filtration of suspensions, the increasing thickness of the formed filter cake and the concomitant resistance gradually decrease the flow rate. Additional difficulties may arise because of the compressibility of biological material. In this case, the resistance offered by the filter cake and, hence, the rate of filtration depend on the pressure applied. If the pressure applied exceeds a certain limit, the cake may collapse and total blockage of the filter can result.
Pressure Filters A filter press (plate filter, chamber filter) is used to filtrate small volumes or to remove precipitates formed during purification. The capacity to retain solid matter is limited, and the method is rather work-intensive. However, these filters are highly suitable for the fine filtration of enzyme solutions.
Vacuum Filters Vacuum filtration is generally the method of choice because biological materials are easily compressible. A rotary vacuum filter is used in the continuous filtration of large volumes. The suspension is usually mixed with a filter aid, e.g., kieselguhr, before being applied to the filter. The filter drum is coated with a thin layer of filter aid (precoat). The drum is divided into different sections so that the filter cake can also be washed and dried on the filter. The filter cake is subsequently removed by using a series of endless strings or by scraper

discharge (knife). The removal of a thin layer of precoat each time exposes a fresh filtering area. This system is useful for preventing an increase in resistance with the accumulation of filter cake during the course of filtration.
Cross-Flow Filtration In conventional methods, the suspension flows perpendicular to the filtering material. In cross-flow filtration, the input stream flows parallel to the filter area, thus preventing the accumulation of filter cake and an increased resistance to filtration. To maintain a sufficiently high filtration rate, thismethodmust consume a relatively large amount of energy, in the form of high flux rates over the membranes. With the membranes now available, permeate rates can be attained. Indeed, in many cases the use of a separator is more economical.



Rotary vacuum filter



Principles of conventional, dead end filtration (A) and cross-flow filtration (B)

The future of this method depends on the development of suitable membranes, but cross-flow filtration can be conveniently used in recombinant DNA techniques to separate organisms in a closed system.

1.2.2 Centrifugation

The sedimentation rate of a bacterial cell with a diameter of 0.5 mmis less than 1 mm/h. An economical separation can be achieved only by sedimentation in a centrifugal field. The range of applications of centrifuges depends on the particle size and the solids content.

Type of centrifuge	Solids content, %	Particle size, µm
Multichamber separator	0–5	0.5–500
Desludging disk separator	β–10	0.5–500
Nozzle separator	5–25	0.5–500
Decanter	5–40	5–50 000
Sieve centrifuge	5–60	5–10 000
Pusher centrifuge	20–75	100–50 000

Decanters (scroll-type centrifuges) work with low centrifugal forces and are used in the separation of large cells or protein precipitates. Solid matter is discharged continuously by a screw conveyer moving at a differential rotational speed.
Tubular bowl centrifuges are built for very high centrifugal forces and can be used to sediment very small particles. However, these centrifuges cannot be operated in a continuous process. Moreover, solid matter must be removed by hand after the centrifuge has come to a stop. A further disadvantage is the appearance of aerosols.
Separators (disk stack centrifuges) can be used in the continuous removal of solid matter from suspensions. Solids are discharged by a hydraulically operated discharge port (intermittent discharge) or by an arrangement of nozzles (continuous discharge). Bacteria and cellular fragments can be separated by a combination of high centrifugal forces, up to 15 000 _ gravity, presently attainable, and short sedimentation distances. Disk stack centrifuges that can be sterilized with steam are used for recombinant DNA techniques in a closed system.

1.2.2 Extraction

An elegant method used to isolate intracellular enzymes is liquid–liquid extraction in an aqueous two-phase system This method is based on the incomplete mixing of different polymers, e.g., dextran and poly(ethylene glycol), or a polymer and a salt in an aqueous solution [208]. The first extraction step separates cellular fragments. Subsequent purification can be accomplished by extraction or, if high purity is required, by other methods. The extractability can be improved by using affinity ligands or modified chromatography gels, e.g., phenyl-Sepharose.

1.2.3 Flocculation and Flotation Flocculation Separation of bacterial cells or cell debris by filtration or centrifugation can involve considerable difficulties due to their small size and physical properties. The compressible nature of the

cells is the primary limiting factor for using filtration as a separation step to remove them. The low permeability of a typical cell cake results in a filtration rate that is often too slow to be practical. In cell removal by centrifugation, the small size and low density difference between the cells or cell debris and the medium results in a low sedimentation rate. Flocculation of cell suspensions has been reported to aid cell separation by both filtration and centrifugation.
Flocculation is the process whereby destabilized particles are induced to come together, make contact, and subsequently form larger aggregates. Flocculating agents are additives capable of increasing the degree of flocculation of a suspension. They can be organic or inorganic, and natural or synthetic. A comprehensive review of various categories of flocculating agents can be found in. Synthetic organic flocculating agents are by far the most commonly used agents for cell flocculation in industrial processes. They are typically water-soluble, charged polymeric substances with average molecular weight ranging from about 10^3 to greater than $5x10^6$ and are generally referred to as polyelectrolytes. The positively and negatively charged polymers are referred to as cationic and anionic polyelectrolytes, respectively. Polyelectrolytes containing both positive and negative charges are termed polyampholytes. Flocculation of cells by polyelectrolytes is a two-step process. The first step is the neutralization of the surface charge on the suspended cells or cell debris. The second step involves the linkage of these particles to form large aggregates. The various mechanisms and theories of flocculation have been summarized. Flocculant selection for a specific cell separation process is a challenge as many factors can impact flocculation. These factors can have their origin in the broth (cell surface charge and size, ionic strength, pH, cell concentration, and the presence of other charged matter), the polymer (molecular weight, charge and charge density, structure, type), and engineering parameters (mixing and mode and order of addition). The final criteria for flocculant selection should take into consideration all aspects of the flocculation process. These include the cost of the added flocculant, subsequent separation performance, process robustness, and yield. In some cases, flocculation can also provide purification by selectively removing unwanted proteins, nucleic acids, lipids and endotoxin from the cell broth.

Flotation If no stable agglomerates are formed, cells can be separated by flotation. Here, cells are adsorbed onto gas bubbles, rise to the top, and accumulate in a froth. An example is the separation of single cell protein.

1.3 Concentration

The enzyme concentration in starting material is often very low. The volume of material to be processed is generally very large, and substantial amounts of waste material must be removed. Thus, if economic purification is to be achieved, the volume of starting material must be decreased by concentration. Only mild concentration procedures that do not inactivate enzymes can be employed. These include thermal methods, precipitation, and to an increasing extent, membrane filtration.

1.3.1Thermal Methods

Only brief heat treatment can be used for concentration because enzymes are thermolabile. Evaporators with rotating components that achieve a thin liquid film (thin-layer evaporator, centrifugal thin-layer evaporator) or circulation evaporators (long-tube evaporator) can be employed.

1.3.2 Precipitation

Enzymes are very complex protein molecules possessing both ionizable and hydrophobic groups which interact with the solvent. Indeed, proteins can be made to agglomerate and, finally, precipitate by changing their environment. Precipitation is actually a simple procedure for concentrating enzymes.
Precipitation with Salts High salt concentrations act on the water molecules surrounding the protein and change the electrostatic forces responsible for solubility. Ammonium sulfate is commonly used for precipitation; hence, it is an effective agent for concentrating enzymes. Enzymes can also be fractionated, to a limited extent, by using different concentrations of ammonium sulfate. The corrosion of stainless steel and cement by ammonium sulfate is a disadvantage, which causes additional problems in wastewater treatment. Sodium sulfate is more efficient from this point of view, but it is less soluble and must be used at temperatures of 35–40 8C. The optimal concentration of salt required for precipitation must be determined experimentally, and generally ranges from 20 to 80 % saturation.
Precipitation with Organic Solvents Organic solvents influence the solubility of enzymes by reducing the dielectric constant of the medium. The solvation effect of water molecules surrounding the enzyme is changed; the interaction of protein molecules is increased; and therefore, agglomeration and precipitation occur. Commonly used solvents are ethanol and acetone. Satisfactory results are obtained only if the concentration of solvent and the temperature are carefully controlled because enzymes can be inactivated easily by organic solvents.
Precipitation with Polymers The polymers generally used are polyethylenimines and poly(ethylene glycols) of different molecular masses. The mechanism of this precipitation is similar to that of organic solvents and results from a change in the solvation effect of the water molecules surrounding the enzyme. Most enzymes precipitate at polymer concentrations ranging from 15 to 20 %.
Precipitation at the Isoelectric Point Proteins are ampholytes and carry both acidic and basic groups. The solubility of proteins is markedly influenced by pH and is minimal at the isoelectric point at which the net charge is zero. Because most proteins have isoelectric points in the acidic range, this process is also called acid precipitation.

1.3.2 Ultrafiltration

A semipermeable membrane permits the separation of solvent molecules from larger enzyme molecules because only the smaller molecules can penetrate the membrane when the osmotic pressure is exceeded. This is the principle of all membrane separation processes, including ultrafiltration. In reverse osmosis, used to separate materials with low molecular mass, solubility and diffusion phenomena influence the process, whereas ultrafiltration and cross-flow filtration are based solely on the sieve effect. In processing enzymes, cross-flow filtration is used to harvest cells, whereas ultrafiltration is employed for concentrating and desalting.

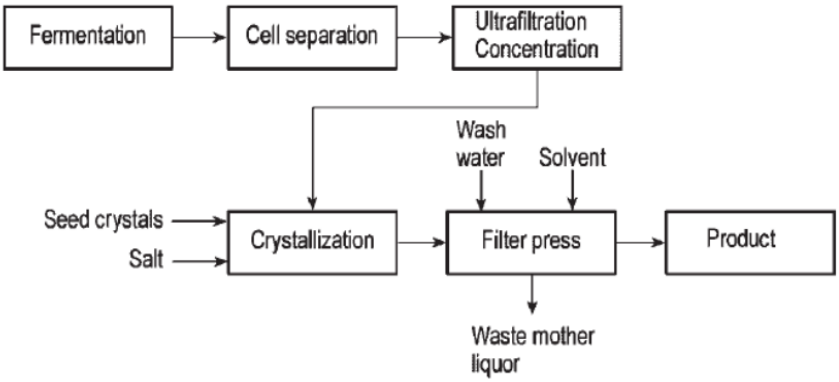
Process	Application	Separation range, M_r
Cross-flow microfiltration	Concentration of bacteria, removal of cell debris	>1 000 000 (or particles)
Ultrafiltration	Concentration of enzymes, dialysis, fractionation	>10 000 (macromolecules)
Reverse osmosis	Concentration of small molecules, desalting	>200

1.4 Purification

For many industrial applications, partially purified enzyme preparations will suffice; however, enzymes for analytical purposes and for medical use must be highly purified. Special procedures employed for enzyme purification are crystallization, electrophoresis, and chromatography.

1.4.1 Crystallization

The rapid growth in the utilization of enzymes in commercial sectors such as agriculture and consumer products requires a cost-effective, industrial-scale purification method. Crystallization, one of the oldest chemical purification technologies, has the potential to fulfill these requirements. Enzyme crystallization is the formation of solid enzyme particles of defined shape and size. An enzyme can be induced to crystallize or form protein-protein interactions by creating solvent conditions that result in enzyme supersaturation. The theory and history of protein crystallization are well documented.Much of the emphasis in enzyme crystallization has focused on obtaining crystals for X-ray diffraction analysis rather than as a purification process.



1.4.2 Electrophoresis

Electrophoresis is used to isolate pure enzymes on a laboratory scale. Depending on the conditions, the following procedures can be used: zone electrophoresis, isotachophoresis, or porosity gradients. The heat generated in electrophoresis and the interference caused by convection are problems associated with a scale-up of this method. An interesting contribution to the industrial application of electrophoresis is a continuous process in which the electrical field is stabilized by rotation.

1.4.3 Chromatography

Chromatography is of fundamental importance to enzyme purification. Molecules are separated according to their physical properties (size, shape, charge, hydrophobic interactions), chemical properties (covalent binding), or biological properties (biospecific affinity). In *gel chromatography* (also called gel filtration), hydrophilic, cross-linked gels with pores of finite size are used in columns to separate biomolecules. Concentrated solutions are necessary for separation because the sample volume that can be applied to a column is limited to ca. 10 % of the column volume. In gel filtration, molecules areseparated according to size and shape. Molecules larger than the largest pores in the gel beads, i.e., above the exclusion limit, cannot enter the gel and are eluted first. Smaller molecules, which enter the

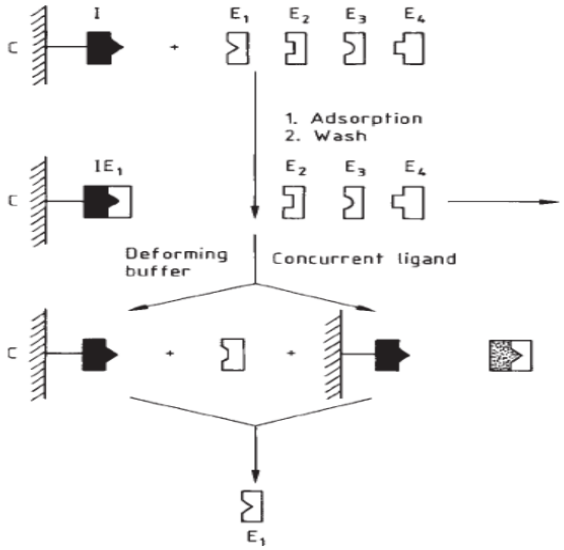
gel beads to varying extent depending on their size and shape, are retarded in their passage through the column and eluted in order of decreasing molecular mass. Gel filtration is used commercially for both separation and desalting of enzyme solutions.

Type of chromatography	Principle	Separation according to
Adsorption	surface binding	surface affinity
Distribution	distribution equilibrium	polarity
Ion exchange	ion binding	charge
Gel filtration	pore diffusion	molecular size, molecular shape
Affinity	specific adsorption	molecular structure
Hydrophobic	hydrophobic chelation	molecular structure
Covalent	covalent binding	polarity
Metal chelate	complex formation	molecular structure

Ion-exchange chromatography is a separation technique based on the charge of protein molecules. Enzyme molecules possess positive and negative charges. The net charge is influenced by pH, and this property is used to separate proteins by chromatography on anion exchangers (positively charged) or cation exchangers (negatively charged). The sample is applied in aqueous solution at low ionic strength, and elution is best carried out with a salt gradient of increasing concentration. Because of the concentrating effect, samples can be applied in dilute form.

For *hydrophobic chromatography*, media derived from the reaction of CNBr-activated Sepharose with aminoalkanes of varying chain length are suitable. This method is based on the interaction of hydrophobic areas of protein molecules with hydrophobicgroups on the matrix. Adsorption occurs at high salt concentrations, and fractionation of bound substances is achieved by eluting with a negative salt gradient. This method is ideally suited for further purification of enzymes after concentration by precipitation with such salts as ammonium sulfate.

In *affinity chromatography*, the enzyme to be purified is specifically and reversibly adsorbed on an effector attached to an insoluble support matrix. Suitable effectors are substrate analogues, enzyme inhibitors, dyes, metal chelates, or antibodies.The insoluble matrix (C) is contained in a column. The biospecific effector, e.g., an enzyme inhibitor (I), is attached to the matrix. A mixture of different enzymes (E_1, E_2, E_3, E_4) is applied to the column. The immobilized effector specifically binds the complementary enzyme. Unbound substances are washed out, and the enzyme of interest (E_1) is recovered by changing the experimental conditions, for example by altering pH or ionic strength.



Immunoaffinity chromatography occupies a unique place in purification technology. In this procedure, monoclonal antibodies are used as effectors. Hence, the isolation of aspecific substance from a complex biological mixture in one step is possible. In this procedure, enzymes can be purified by immobilizing antibodies specific for the desired enzyme.Amore generalmethod offers the synthesis of a fusion proteinwith proteinAby ‘‘protein engineering’’. Protein A is a Staphylococcus protein with a high affinity formany immunoglobulins, especially of the IgG class of antibodies. In this way, enzymes that usually do not bind to an antibody can be purified by immunoaffinity chromatography.

Covalent chromatography differs from other types of chromatography in that a covalent bond is formed between the required protein and the stationary phases.

Immobilization of enzymes

The term immobilized enzymes refers to "enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously". Immobilized enzymes are currently the subject of considerable interest because of their advantages over soluble enzymes. In addition to their use in industrial processes, the immobilization techniques are the basis for making a number of biotechnology products with application in diagnostics, bioaffinity chromatography, and biosensors. At the beginning, only immobilized single enzymes were used, after 1970s more complex systems including two-enzyme reactions with cofactor regeneration and living cells were developed. The enzymes can be attached to the support by interactions ranging from reversible physical adsorption and ionic linkages to stable covalent bonds. Although the choice of the most appropriate immobilization technique depends on the nature of the enzyme and the carrier, in the last years the immobilization technology has increasingly become a matter of rational design. As a consequence of enzyme immobilization, some properties such as catalytic activity or thermal stability become altered. These effects have been demonstrated and exploited. The concept of stabilization has been an important driving force for immobilizing enzymes.

Technological properties of immobilized enzyme systems

Advantages	Disadvantages
Catalyst reuse	Loss or reduction in activity
Easier reactor operation	Diffusional limitation
Easier product separation	Additional cost
Wider choice of reactor	

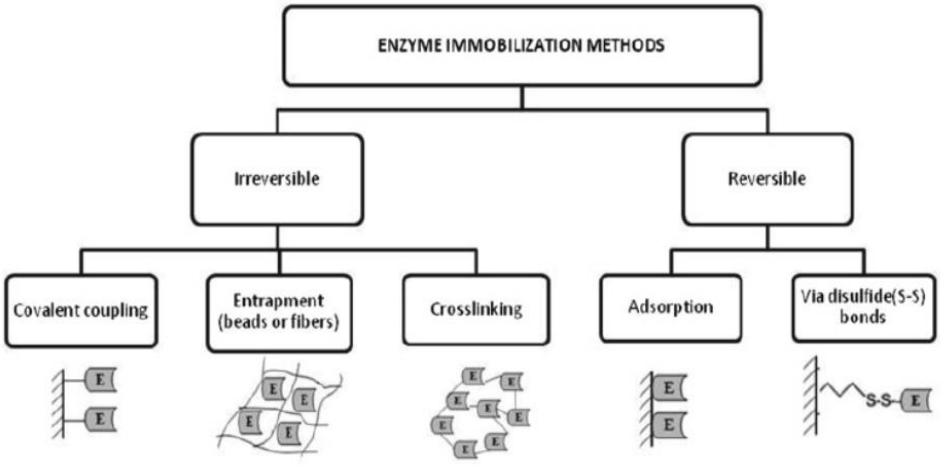
Major products obtained using immobilized enzymes

Enzyme	Product
Glucose isomerase	High-fructose corn syrup
Amino acid acylase	Amino acid production
Penicillin acylase	Semi-synthetic penicillins
Nitrile hydratase	Acrylamide
β-Galactosidase	Hydrolyzed lactose (whey)

Steps in the development of immobilized enzymes

Step	Date	Use
First	1815	Empirical use in processes such as acetic acid and waste water treatment.
Second	1960s	Single enzyme immobilization: production of L-amino acids, isomerization of glucose, etc.
Third	1985–1995	Multiple enzyme immobilization including cofactor regeneration and cell immobilization. Example: production of L-amino acids from keto-acids in membrane reactors.
Fourth	1995 to present	Ever-expanding multidisciplinary developments and applications to different fields of research and industry.

Methods of Immobilization



Advantages and disadvantages of the main enzyme immobilization methods

Methods and binding nature	Advantages	Disadvantages
Physical adsorption Weak bonds: hydrophobic, Van der Waals or ionic interactions.	Simple and cheap Little conformational change of the enzyme	Desorption Nonspecific adsorption
Affinity Affinity bonds between two affinity partners	Simple and oriented immobilization Remarkable selectivity	High cost
Covalent binding Chemical binding between functional groups of the enzyme and support	No enzyme leakage Potential for enzyme stabilization	Matrix and enzyme are not regenerable Major loss of activity
Entrapment Occlusion of an enzyme within a polymeric network	Wide applicability	Mass transfer limitations Enzyme leakage
Cross-linking Enzymes molecules are cross-linked by a functional reactant	Biocatalyst stabilization	Cross-linked biocatalysts are less useful for packed beds. Mass transfer limitations Loss of activity

Methods of Irreversible Enzyme Immobilization

The concept of irreversible immobilization means that once the biocatalyst is attached to the support, it cannot be detached without destroying either the biological activity of the enzyme or the support. The most common procedures of irreversible enzyme immobilization are covalent coupling, entrapment or microencapsulation, and cross-linking.

Formation of Covalent Bonds

Immobilization of proteins by methods based on the formation of covalent bonds is among the most widely used. An advantage of these methods is that, because of the stable nature of the bonds formed between enzyme and matrix, the enzyme is not released into the solution upon use. However, in order to achieve high levels

of bound activity, the amino acid residues essential for catalytic activity must not be involved in the covalent linkage to the support, and this may prove a diffi cult requirement to fulfill in some cases. A simple procedure that sometimes improves the activity yield is to carry out the coupling reaction in the presence of substrate analogues. Covalent methods for immobilization are employed when there is a strict requirement for the absence of the enzyme in the product.

Entrapment and Cross-linking

The entrapment method is based on the occlusion of an enzyme within a polymeric network that allows the substrate and products to pass through but retains the enzyme. This method differs from the coupling methods described above, in that the enzyme is not bound to the matrix or membrane. There are different approaches to entrapping enzymes such as gel or fiber entrapping , and micro-encapsulation. The practical use of these methods is restricted by mass transfer limitations through membranes or gels. The more recently reported technique for immobilization of enzymes as cross-linked enzyme aggregates (CLEAs) diverges slightly from the conventional immobilization methods. CLEAs are based on multipoint attachment through intermolecular cross-linking between enzyme molecules. Successful preparation of CLEAs from a broad range of enzymes, including penicillin acylases, lipases, laccases, and horseradish peroxidase is currently being evaluated by many researchers.

Methods of Reversible Immobilization

Because of the type of the enzyme-support binding, reversibly immobilized enzymes can be detached from the support under gentle conditions. The use of reversible methods for enzyme immobilization is highly attractive, mostly for economic reasons simply because when the enzymatic activity decays the support can be regenerated and re-loaded with fresh enzyme. Indeed, the cost of the support is often a primary factor in the overall cost of immobilized catalyst. The reversible immobilization of enzymes is particularly important for immobilizing labile enzymes and for applications in bioanalytical systems.

Nonspecific Adsorption

In physical adsorption the enzymes are attached to the matrix through hydrogen bonding, van der Waals forces, or hydrophobic interactions, whereas in ionic bonding the enzymes are bound through salt linkages. The nature of the forces involved in noncovalent immobilization results in a process which can be reversed by changing the conditions that infl uence the strength of the interaction (pH, ionic strength, temperature, or polarity of the solvent). Immobilization by adsorption is a mild, easy to perform process, and usually preserves the catalytic activity of the enzyme. Such methods are therefore economically attractive, but may suffer from problems such as enzyme leakage from matrix when the interactions are relatively weak.

Ionic Binding

An obvious approach to the reversible immobilization of enzymes is to base the protein-ligand interactions on principles used in chromatography. For example, one of the fi rst applications of chromatographic principles in the reversible immobilization of enzymes was the use of ion-exchangers. The method is simple and reversible but, in general, it is diffi cult to fi nd conditions under which the enzyme remains both strongly bound and fully active. More recently, the use of immobilized polymeric ionic ligands has allowed to modulate the interactions between protein and matrix and thus to optimize the properties of the derivative. A number of patents have been fi led on the use of polyethyleneimine to bind a rich variety of enzymes and whole cells.

Hydrophobic Adsorption

Another approach is the use of hydrophobic interactions. In this method, it is not the formation of chemical bonds but rather an entropically driven interaction that takes place. Hydrophobic adsorption has been used as a chromatographic principle for more than three decades. It relies on well-known experimental variables such as pH, salt concentration, and temperature. The strength of interaction relies both on the hydrophobicity of the adsorbent and that of the protein. The hydrophobicity of the adsorbent can be regulated by the degree of substitution of the support and by the size of the hydrophobic ligand molecule.

Affinity Binding

The principle of affi nity between complementary biomolecules has been applied to enzyme immobilization. The remarkable selectivity of the interaction is a major benefi t of the method. However, the procedure often requires the covalent binding of a costly affinity ligand (e.g., antibody or lectin) to the matrix.

Formation of Disulfide Bonds

These methods are unique because, even though a stable covalent bond is formed between matrix and enzyme, this bond can be broken by reaction with a suitable agent such as dithiothreitol (DTT) under mild conditions. Additionally, since the reactivity of the thiol groups can be modulated by changing the pH, the activity yield of the methods involving disulfide bond formation is usually high, provided that an appropriate thiol-reactive adsorbent with high specificity is used.

Properties of Immobilized Enzymes

The properties of immobilized enzymes are determined by the characteristics of carrier material as well as by the nature and number of interactions between the enzyme and the support. As a consequence of enzyme immobilization, the stability and kinetic properties of enzymes are usually changed, mostly due to the microenvironment and modifi cations imposed by the supporting matrix. This modifi cation in the properties may be caused either by changes in the intrinsic activity of the immobilized enzyme or by the fact that the interaction between the immobilized enzyme and the substrate takes place in a micro-environment that is different from the bulk solution. So, one of the main problems associated with the use of immobilized enzymes is the loss of catalytic activity, especially when the enzymes are acting on macromolecular substrates. Because of the limited access of the substrate to the active site of the enzyme, the activity may be reduced to accessible surface groups of the substrate only. This steric restriction may in turn, change the characteristic pattern of products derived from the macromolecular substrate.

Applications of Immobilized Enzymes

Immobilized enzymes have been widely studied during the last few decades. Biocatalyst systems may work as biosensors or may be used for the treatment of different diseases. This chapter presents different attempts to immobilize enzymes in the biomedical field, particularly the use of prolidase and superoxide dismutase as two examples of this approach. Although this chapter focuses on liposomes and nanoparticles for the entrapment of these enzymes, the methods detailed here could be adapted for the immobilization of other enzymes with therapeutic purposes.

Enzyme	Disease treated
Adenosine deaminase	SCID
Alcohol dehydrogenase and acetaldehyde dehydrogenase	Alcohol intoxication
Arginine deiminase	Human melanoma and hepatocarcinoma
Cytochrome P450 (cells producing the enzyme)	Cancer therapy, to convert ifosfamide to its cytotoxic metabolite
Deoxyribonuclease I	Cystic fibrosis
Fibrinolytic enzyme	Cardiovascular therapy
Glucose oxidase-peroxidase	Oral infections
L-asparaginase	Leukemia
Organophosphorous hydrolase	Organophosphate intoxication
Pepsin, chymotrypsin, trypsin	Replacement therapy in gastrointestinal diseases, treatment of fat malabsorption
Phenylalanine ammonia lyase	Phenylketonuria
Prolidase	Prolidase deficiency
Streptokinase	Thrombolytic therapy
Thymidine phosphorylase	Mitochondrial Neurogastrointestinal Encephalomyopathy (MNGIE)
Tissue plasminogen activator	Thrombolytic therapy
Trypsin and urokinase	Thrombolytic therapy
Urease (*E. coli* cells engineered to produce urease)	Removal of urea in kidney failure
β-Glucosidase	Gaucher's disease

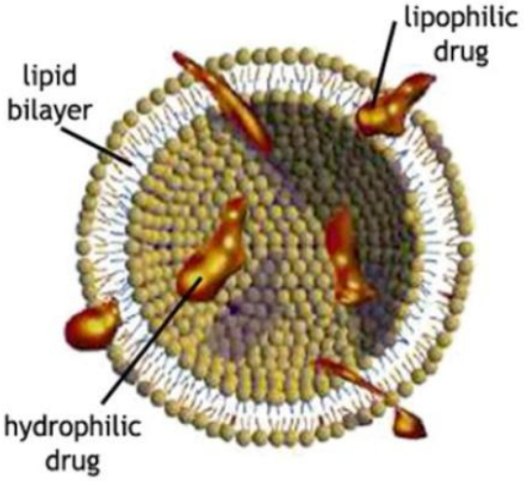
Many approaches have been developed in order to achieve enzyme immobilization. Among them, two main strategies have arisen, the first one is based on binding the enzyme, either covalently or by adsorption to a support (***immobilization by binding***) and the second one consists of entrapping the enzyme into a matrix (***immobilization by entrapment***)

Regarding *immobilization by binding*, two main methods can be distinguished, cross-linking and support-based immobilization. In the early 1960s, researchers discovered that by mixing enzymes and a cross linker active aggregates were formed. CLE (Cross-Linked Enzyme), CLEC (Cross-Linked Enzyme Crystals), and CLEA (Cross-Linked Enzyme Aggregate) are the best known enzyme cross-linked product. The difference between them lies in the state that enzyme presents prior to cross linking process, i.e., it is dissolved, in crystals or aggregated. CLECs show improved thermal and mechanical stability, broader pH stability and withstanding of organic solvents and the possibility of controlling size from 1 to 100 μ m, compared to CLE. However, the crystallization of enzyme is quite a laborious step. In order to avoid the purifying and crystallization steps, CLEAs were developed and therefore the elaboration technique was greatly simplified.

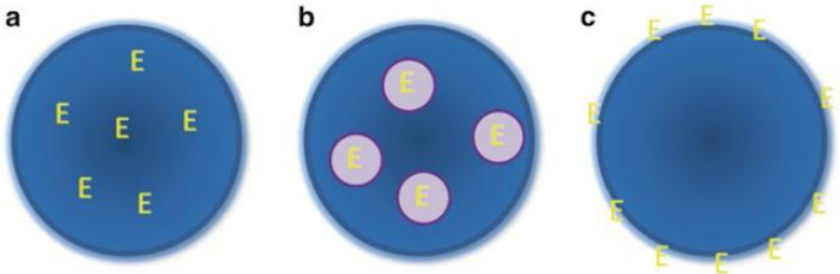
When *enzyme entrapment* is employed, no chemical reaction involving the enzyme itself is undergone. Moreover, protection level and enzyme loading is substantially enhanced in these systems. The enzyme is enclosed in a wide range of polymers. The entrapment can be achieved by holding into synthetic polymer, biodegradable polymers (polymers and copolymers derived from lactic and glycolic acid, alginate, chitosan, etc.) or by the use of other biocompatible materials like liposomes or even red blood cells.

Liposomes are vesicles formed by phospholipid bilayer in the nanometric range. This drug delivery system has been extensively studied over the last few decades. As a consequence, different liposome formulations are currently commercially available, such as AmBisome® or DOXIL. Liposomes enclose hydrophilic drugs in its inner aqueous spaces and thus prolong in vivo circulation time and what is more, they may enhance targeting to specific body sites by ligand coupling. In addition, as the enzyme is encapsulated inside the vesicle its antigen determinants are masked from the immune system.

Structure of a liposome. Hydrophilic drugs are encapsulated in the inner aqueous space, whereas lipophilic drugs are entrapped in the phospholipid membrane



Another drug delivery system approach spans the use of polymeric nano/microparticles. For this encapsulation attempt biodegradable polymers can be used in order to obtain particles above 10 nm. In these colloidal systems the drug can be dissolved, entrapped or adsorbed. Nanoparticles display some advantages for enzyme delivery due to their controlled release capability, formulation versatility, sub cellular size and biocompatibility.



Drug delivery system for enzyme (E: enzyme), microparticles and nanoparticles. Immobilized enzyme may be dispersed (**a**), dissolved (**b**), or adsorbed (**c**) into the polymers forming the particle

Another application field of immobilized enzymes lies on the use of enzymes with antioxidant properties. Increased reactive oxygen species (ROS) involve cell and tissue damage, being this accumulation produced due to oxidative stress occurring in many different situations and illnesses. ROS could be neutralized by antioxidant enzymes, mainly by superoxide dismutase (SOD). ROS are associated to a wide range of illnesses where an inflammatory pathology underlies, for instance atherosclerosis, Parkinson's disease, autoimmune disorders, or cancer.

ABZYMES

An **abzyme** (from antibody and enzyme), also called *catmab* (from *catalytic monoclonal antibody*), is a monoclonal antibody with catalytic activity. Molecules which are modified to gain new catalytic activity are called synzymes. Abzymes are usually artificial constructs, but are also found in normal humans (anti-vasoactive intestinal peptide autoantibodies) and in patients with autoimmune diseases such as systemic lupus erythematosus, where they can bind to and hydrolyze DNA. Abzymes are potential tools in biotechnology, e.g., to perform specific actions on DNA.

Enzymes function by lowering the activation energy of the transition state, thereby catalyzing the formation of an otherwise less-favorable molecular intermediate between reactants and products. If an antibody is developed to a stable molecule that's similar to an unstable intermediate of another (potentially unrelated) reaction, the developed antibody will enzymatically bind to and stabilize the intermediate state, thus catalyzing the reaction. A new and unique type of enzyme is produced.

Reference

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