LESSON 21:
IMMOBILIZATION OF ENZYMES

What do You Think is the Need of Immobilizing the Enzymes

The objective of immobilization is the economic application of enzyme systems. Other benefits such as ease of control and uniformity of conversions may be derived from immobiliza-tion tech-niques. The greatest return from immobilization is achieved with expensive enzymes since there is a definitive cost associated with immobilizing processes. Hence enzymes that are more expensive to produce, includ-ing many intracellular microbial enzymes, and those employed in biosensors and analytical test systems, are often used in an immobilized form. It is clearly apparent. then, that a very inexpensive enzyme should not be employed as an immobilized derivative in a process unless another advantage such as avoidance of contami-nation or immune response is attained.

The process of enzyme immobilization is physically confining or localizing the biocatalyst in a certain defined region of space with retention of its catalytic activity, which can be used repeatedly and continuously.

Beneficial Feature of Immobilized Enzyme

In industrial processes (Table), immobilization enzyme preparations have several beneficial features, including:

1. reuse
2. suitability for application within continuous operations
3. their product is enzyme free, therefore further processing to remove or inactivate the enzyme is not required
4. improved enzyme stability
5. reduced effluent disposal problems.

Table : Some important industrial microbial enzymes that are used in an immobilized form

Enzyme	Source	Product/role
Aminoacylase	Aspergillus oryzae	L-amino acids
Amyloglucosidase	Aspergillus niger Rhizopus niveus	glucose production from starch
Glucose isomerase	Actinomyces missouriensis	high fructose corn syrup
Bacillus coagulans		
Hydamoinase	Flavobacterium ammoniagenes	D- and L-amino acids
Invertase	Saccharomyces cerevisiae Aspergillus niger	invert sugar (glucose + fructose)
Lactase	Aspergillus oryzae Kluyveromyces fragili	lactose-free milk and whey
Lipase	Rhizopus arrhizus	cocoa butter substitutes
Naringinase	Penicillium decumbens	debittering of citrus fruit juice
Nitrile hydratase	Rhodococcus rhodochrous	acrylamide
Penicillin acylase	Escherichia coli Bacillus subtilis	penicillin side chain cleavage
Melibiase (raffinase)	Aspergillus niger	removal of raffinose from sugar beet extracts
(a-galactosidase)	Saccharomyces cerevisiae	
Thermolysin	Bacillus thermoproteolyticus	Aspartame (L-aspartyl-L-phenylalanine methyl ester) a low-calorie sweetener

Techniques of Enzyme Immobilization

There are five markedly different techniques of immobilizing enzymes:

1. adsorption of the enzyme on a carrier surface
2. ionic bonding or covalent coupling of an enzyme to a carrier surface ie. inert inorganic or organic solid support ma-terials, such as nylon, bentonite, cellulose and dextran.
3. crosslink-ing between enzyme molecules (copolymerization);
4. entrapment of an enzyme in a matrix ie. within gels or fibres, and intracellular enzymes may be immobilized within their producer cells.
5. encapsulation or confinement of an enzyme solution in a membrane structure.

Although each immobilization technique is unique, they are by no means pure processes and are in combination with other methods.

- Adsorption on a car-rier surface, either intentionally or unintentionally, will involve crosslinking between enzyme molecules to some extent.
- Covalent coupling to a carrier surface usually involves adsorption of enzymes on that surface and crosslinking between enzyme molecules.
- Entrapment of an enzyme in a matrix may involve adsorption, covalent coupling to the surface or crosslinking between the enzyme molecules.
- Encapsulation or confinement of an enzyme solution within a membrane structure minimizes contamination by these mixed immobilization effects, however, some adsorption on the membrane surface and some crosslin king of the molecules do occur.

Each immobilization process has both advantages and disadvantages. The choice of technique, therefore, should be decided by the specific con-ditions of the application which would selectively employ the positive attributes of the specified immobilization.

Historical Aspect of Enzyme Immobilization

Getting into the history of enzyme immobilization, Nelson and Griffin in 1916 found accidentally that yeast invertase adsorbed on activated charcoal catalyses the hydrolysis of sucrose. This experiment was the point of initiation of enzyme immobilization process. However enzyme immobilization came into commercial practice only when Grubhofer and Schleith immobilized various enzymes like carboxypeptidase, diastase, pepsin and ribonuclease. The enzymes were immobi-lized on diazotised polyaminostyrene resin by covalent binding. Since then different principles of immobilization has been tried and developed which includes:

- Ionic binding
- Physical adsorption
- Entrapment.

The first industrial application of immobilized enzyme was performed by Chibata and coworkers in 1969 when they used fungal aminoacylase immobilized on DEAE Sephadex through ionic binding. The immobilized enzyme was used for the hydrolysis of N-acyl-D, L-amino acids and N-acyl-D-amino acids.

Enzyme Immobilization Methods

Before we discuss different methods of immobilization we need to clear the concept of what are Carriers.

Definition of a Carrier

A carrier is defined as the support material utilized to immobilize the enzyme. This support may be a matrix system, a membrane or a solid surface.

Carrier Durability under Application Conditions

- When a carrier is employed for the immobilization of an enzyme, the durability of that carrier during use is second in importance only to the enzyme activity.
- If the carrier, whether it be a matrix or a membrane, is not stable under the pH, ionic strengths and solvent conditions of the application, the carrier will be disrupted or dissolved and the enzyme will be released into solu-tion.
- This does not necessarily mean that the carrier must be durable under all pH, ionic strengths and solvent conditions, but only those of the specific application.
- In addition to the chemical environment a most important consideration is that of the physical environment of the application. A rigid structure, such as an inorganic material, may be readily abraded in a continuous stirred reactor. This will not only lead to dissolution of the enzyme but also to formation of non-uniform particles which may disrupt the reactor performance. Under these conditions it would be far more advantageous to choose an elastic type carrier for this application.

Methods of Immobilization

Immobilization methods can be grouped into following broad categories

1. Carrier binding
2. Cross linking
3. Entrapping

The choice of methods is based on the enzyme, reaction and the reactor.

The desired properties of carrier are:

a. Should have adequate functional groups for immobilization
b. Mechanical strength
c. Physical, chemical and biological stability
d. Non toxic

Carrier Binding Method

- Based on binding of the biocatalyst to a water insoluble carrier through—
  a. Covalent linkage
  b. Ionic bonds
  c. Physical adsorption
  d. Biospecific binding
- Carrier used after proper modification or activation
  a. Polysaccharides (cellulose, dextran and agarose derivative)
  b. Proteins (gelatin, albumin)
  c. Synthetic polymers (polystyrene derivatives, ion exchange resins and polyurethane)
  d. Inorganic materials (brick, sand, ceramics and magnetite)

A. Covalent Binding

- Covalent attachment to surfaces generally offers the advantage of an immobilized enzyme sys-tem that can be utilized under a broad spectrum of pH conditions, ionic strengths and uncon-trolled variable conditions.
- The disadvantage of this type of attachment is that generally it requires a multistep process to immobilize the enzyme. These steps may include the attachment of a coupling agent following by an activation process or the attachment ‘of a functional group, and finally the attachment of the enzyme.
- Covalent attachment may be directed to a specific group (amine, hydroxyl, tyrosyl, *etc.)* on the surface of the enzyme. Thus, the active site of the enzyme can be avoided by judiciously choosing a group on the surface of the enzyme protein that is not involved in the site.
- Frequently, covalent coupling is preferred to other processes where the enzyme may contain polymeric units or prosthetic groups. There appears to be fewer tendencies to disrupt the complex nature of these enzymes since specific bonds can be formed with the functional group. .
- Enzyme composites may be formed with covalent techniques that are appropriately tailored for specific substrates or environments. For instance, if the substrate molecule is hydrophobic and aliphatic, then it would be more compatible with an aliphatic environment and a long chain cou-pling agent may be attached to the carrier prior to the attachment of the enzyme. On the other hand, if the substrate is hydrophic and aromatic, then an aromatic coupling agent may be attached to the surface so that it will increase the compatibility for the enzyme-substrate reaction. The hydrophobic composite may additionally be required for enzyme reactions occurring in partially organic solvent environments. If a hydrophilic environment is required, then a coupling agent containing a number of hydroxyl groups may be utilized.

The covalent-bindng method has the following

Advantages

i. The enzyme does not leak or detach from carrier.

ii. The biocatalyst can easily interact with the substrate because it is on the surface of the carrier

iii. The biocnalvst stability is often increased because of the strong interaction between it and the carrier.

Disadvantages

i. The activity yield is likely to be low owing to exposure of the biocatalyst to toxic reagents or severe reaction conditions.

ii. The optimal conditions for the immobilization procedure are difficult to find

iii. Renewal of the carrier and recovery of the bio-catalyst from the carrier are, in general, impossible

Hence, this method is better suited to expensive enzyme whose stability is significantly improved by covalent binding to the carrier.

B. Ionic Binding Method

- Since catalase was found to be able to bind to the ion-exchange cellulose the ionic binding method has been applied for the immobilization of many biocatalysts.
- The procedure is simple, renewal of the carrier and recovery of the biocatalyst from the carrier are easy, and the conditions of immobilization are mild.
- The first industrial application of immobilized enzymes employed this method for the immobdizarion of aminoacylase on DEAE-Sephadex to produce L.-amino acids.
- In ionic binding, binding of the biocatalyst to the carrier is affected by
  a. buffer used
  b. pH
  c. ionic strength
  d. temperature.
- Although renewal of the carrier and recovery of the bio-catalyst from the carrier are easy, the biocatalyst is likely to detach from the carrier.
- The polysaccharide derivatives having ion-exchange groups, as well as various ion exchange resins, can be utilized for this purpose.

C. Physical Adsorption Method

- Although operationally adsorption is the most economical and simple process to perform, the forces involved are the most complex.
- The surface activity of the support acting in concert with a functional moiety or characteristic group on the surface of the enzyme protein is responsible for the bonding or immobilization of the enzyme.
- The bonds that exist between the enzyme protein and the carrier depend upon the nature of the carrier and the nature of the enzyme protein surface.
- These bonds may be ionic, hydrogen, coordinate covalent, covalent, hydrophobic or a combination of any of the aforementioned bonds.
- An enzyme may be immobilized by bonding to either the external or internal surface of a carrier.
- If the enzyme is immobilized externally, the carrier particle size must be very small in order to achieve an appreciable surface for bonding. These particles may have diameters, which range from 500 Å to about 1 mm.
- The advantage of immobilizing the enzyme on an external surface is that no pore diffusion limitations are encountered.
- The disadvantages of external adsorption are: (1) a relatively low surface area for bonding; (2) the enzyme is more subject to physical abrasion, inhibitory effects and the turbulence of the bulk solution; (3) the enzyme is more exposed to microbial attack; and (4) smaller particles result in high pressure drops in a continuous packed bed reactor and are difficult to retain in fluidized beds or continuous-stirred reactors.
- Immobilization of an enzyme to the internal surface of a porous carrier has one marked disadvantage however many advantages may be harvested from this approach. The major disad-vantage of internal pore immobilization is that of pore diffusion limitations. If the pore diameter is optimized with respect to either the enzyme molecule or the substrate, whichever is the larger, then internal immobilization offers several more magnitudes of surface area for the immobiliza-tion of the enzyme than can be achieved with very small particles by external immobilization.
- Unlike the situation resulting from external immobilization, the enzyme immobilized on an inter-nal surface is protected from abrasion, inhibitory bulk solutions and microbial attack. Generally speaking, a more stable and active enzyme system may- be achieved with internal pore immobili-zation.
- Three forces involved in internal surface immobilization are an attractive surface force, diffusion and the formation of multiple hydrogen bonds.
- The effective bonding surface appeared to be a function of the pore diameter and the molecular weight of the protein in internal immobilization. To utilize fully the internal surface of porous carriers for attaching the enzyme and yet maintain as high a surface area as possible the smallest pore diameter with a very narrow pore distribution is required that entry of the limiting molecule (the enzyme when it is larger than the substrate) is just possible.

Modification and Preconditioning of Carrier Materials Before Immobilization

The carriers generally used for immobilization are very active materials, A major concern should be that these materials are thoroughly cleaned and fully activated with respect to their surfaces prior to the exposure of the enzyme solution. Carrier materials will adsorb volatile organic substances from the air, as well as microbes. These contaminants will mask the functional groups on the surface and thus prevent the immobilization of the enzyme. The cleaning processes for the specific carrier will depend upon the basic durability characteristics of that carrier. A

carrier that is stable in an cid environment may be cleaned with an acid, and that in basic environment may be cleaned with a base. Few organic solvents (alcohol, acetone or carbon tetrachloride) may be employed for the removal of fine organic films.

Inorganic carrier surfaces are cleaned by exposure of the carrier to temperatures in excess of 450°C in the presence of sufficient air or oxygen to volatilize the carbonaceous material.

Each enzyme has its own stability characteristics with respect to pH, ionic strengths, activators and cofactors. Hence preconditioning of the carrier surface for the enzyme is necessary. Carriers appear to selectively bind such cofactors as metal ions. The carrier, therefore, is in competition with the enzyme requiring metal ions for activation. These metal ions are frequently removed from the functional site of the enzyme and are bound to the carriers. Hence the enzyme becomes inactive. If the carrier is exposed to a solution of the appropriate metal ions required for this enzyme, the resultant preparation will be fully active.

Immobilization Methods by Adsorption

There are four procedures that have been used for the immobilization of enzymes by adsorp-tion. They are the

a. static_process
b. dynamic batch process
c. reactor loading process
d. electrodeposition process

a. The Static Process

- The enzyme is immobilized on the carrier by simply allowing the solution containing the enzyme to contact the carrier without agitation or stirring.
- Generally, the loading of the enzyme on the car-rier, surface is not uniform and is rather low.
- The carrier must be either exposed to high concen-trations of the enzyme or,for long periods of time at lower concentrations in order to achieve a active immobilized enzyme preparation.-

b. The Dynamic Batch Process

- The_carrier is placed into the enzyme solution and the carrier and enzyme solution, are either mixed_by stirring. or agitated continuously on a shaker.
- This process is rather effective and normally result in relatively uniform high loading if adequate concentrations of. enzymes are employed.

Precautions should be taken to ensure that agitation is not so vigorous that the carrier will be abraded and disrupted, however, it needs to be sufficiently vigorous to allow a low density carrier surface to be adequately exposed to the enzyme solution.

c. The Reactor Loading Process

- The carrier is placed into the reactor along with the enzyme.
- The carrier is loaded in a dynamic environment by either circulating the enzyme or by agitation of the enzyme solution and carrier.

d. The Electrodeposition Process

- Electrodeposition is a process in which the carrier is placed proximal to one of the electrodes in an enzyme bath, the current is turned on, the enzyme migrates to the carrier and is deposited upon the surface.
- It should be ascertained prior to the employment of a specific carrier whether that particular carrier is durable in an electric field.
- In an electric field, ions will be removed from the surface of the carrier.
- If these ions are involved in the stability or the activity of the enzyme, then either the ions should be added to the bath solution or replaced iri the immobilized prep-aration as soon as possible.

D. Biospecific Binding Method

- The biospecific binding method is based on the biospecific interaction between the enzymes and other substances such as coenzymes, inhibitors, effectors, lectins, and antibodies, which are often utilized for affinity separation pro-cesses.
- If the interaction is strong, the enzyme can he immobilized on the carrier conjugated with one of these substances.
- Antibodies and inhibitors are however are not good choices because the enzyme is usually inactivated by binding to them.

Interaction between a lectin and the carbohohydrate moiety of an enzyme is useful for this application.

Cross-linking Method

- The cross-linking method utilizes a bi- or multifunctional compound as in the carrier cross-linking method; however, a carrier is not used in this method.
- The bi- and multi-functional compound serves as the reagent for intermo-lecular cross-linking of the biocatalyst.
- The cross-linked biocatalyst becomes water insoluble.
- The activity of the biocatalyst immobilized by this method is, in general, reduced.

Cross Linking Reagents

- glutaraldehyde
- toluene diisocyanate
- hexamethylene diisocyanate

Method of Crosslinking of Catalase

- Crystalline catalase is dissolved in 10% NaCI and diluted with 0.05 M phosphate buffer (pH 7.2) until the concentration of catalase is 2 mg/ ml.
- A solution of 4% glutaraldehyde (4 ml) in the same buffer is added to 4 ml of the enzyme solution and stirred for about 1 h at room temperature, until green lumpy precipitates appear.
- This cross-linking reaction can be performed in the cold room overnight with comparable results.
- The precipitates are separated by centrifugation (5 min, 4,000 rpm) and washed repeatedly with a 10% NaCI

solution (6 to 8 times) until the supernatant fluid is free of catalase activity.

- The pre-cipitates are homogenized in water by a Teflon pestle, to a fine suspension containing 1 mg/ ml of the immobilized cat-alase.

Entrapment Method

- The entrapment method is classified into five major types: lattice, microcapsule, liposome, membrane, and reversed micelle.
- In the lattice type, the biocatalyst is entrapped in the matrix of one of the various polymers.
- The microcapsule type involves entrapment within microcapsules of a semi-permeable synthetic polymer.
- The liposome type employs entrapment within an amphiphatic liquid-surfactant membrane prepared from lipid.
- In the membrane type, the biocatalyst is separated from the reaction solution by an ultrafiltration membrane, a microfiltration membrane, or a hollow fiber.
- In the reversed micelle type, the biocatalyst is entrapped within the reversed micelles, which are formed by mixing a surfactant with an organic solvent.

Advantage

Not only single enzymes but also multiple enzymes, cellular organelles, and intact or treated cells can be immobilized.

Disadvantages

i. High-molecu-lar-weight substrate may not be able to access the en-trapped biocatalyst

ii. renewal of the carrier is difficult.

Among the entrapment methods, the lattice type is the most widely used. Several representative techniques for the lattice-type method are described.

A. Polyacrylamide Gel Method

- Bernfeld and Wan reported the entrapment of several enzymes in polyacrylamide gel in 1963.
- Various types of biocatalyst, including cellular organelles, micro-bial cells, plant cells and animal cells, have been immo-bilized by this method.
- This method has also been applied to the industrial production of L-aspartate, L-malate, and acrylamide.
- The procedure for preparation of the gel is identical to that employed for electrophoresis.
- For the immobilization of the biocatalyst by this method, acrylamide and N,N' -methylenebisacryl-amide (BlS) as the cross-linking reagent are mixed with the biocatalyst and polymerized in the presence of an ini-tiator, such as potassium persulfate, and a stimulator, such as 3-dimethylaminopropionitrile (DMAPN) or N,N,N',N'-tetramethylethylenediamine (TEMED).

Disadvantages

- toxicity of the acrylamide mon-omer, the cross-lmking reagent, the initiator, and the stim-ulator.
- In some cases, free-radical polymerization results in a decrease in the activity of the biocatalyst.

Method for Entrapment of E. coli with a Polyacryl-Amide Gel

- In 4 ml of physiological saline, 1g of packed intact E. *coli* cells are suspended.
- To the suspension, acrylamide monomer (0.75 g), BIS (40 mg), 5% DMAPN (0.5 ml), and 2.5% potassium persulfate (0.5 ml) are added, and the mixture is incubated at 37°C for 30 min to yield the polyacrylamide gel containing the cells.
- Polymerization should be carried out under anaerobic conditions, because oxygen prevents the polymerization.
- The gel is washed with physiological saline after being made to the proper shape.

B. Alginate Gel Method

- Several natural polysaccharides, such as alginate, K-carra-geenan, agar, and agarose, are excellent gel materials and are used widely for entrapment of biocatalysts.
- Alginate is a linear copolymer of D-mannuronic and L-guluronic acids and can be gelled by multivalent ions.
- For immobilization of the biocatalyst, sodium alginate, which is soluble in water, is mixed with a solution or suspension of the biocatalyst and dropped into a calcium chloride so-lution to form the water-insoluble calcium alginate gel droplets.
- Calcium alginate gels however are gradually solubilized in the presence of a calcium ion-trapping rea-gent such as phosphate ion.
- Treatment of the calcium alginate gel with a cationic polymer such as polyethyleneimine can improve the stability of the gel in the presence of phosphate.
- It is also known that addition of polyacryl acid to sodium alginate increases the physical strength of the gel and pre-vents the gel from being dissolved by bridging the poly-acrylate chains and alginate chains with calcium ions.

Methods of Entrapment of S. Cerevisiae in Calcium Alginate

- S. cerevisiae (25 g wet weight) is suspended in sterile tap water and mixed with 50 ml of 4% sodium alginate.
- The resulting suspension is passed through a narrow tube of about 1 mm diameter and dropped into a calcium chloride solution (50 mM, pH 6 to 8).
- The beads (2.8 to 3.0 mm diameter) obtained are in-cubated in the calcium chloride solution at 20 to 22°C for 2 h to harden the gel.

C. K-Carrageenan Gel Method

- K-Carrageenan is a readily available, nontoxic polysaccha-ride, which is obtained from seaweed. It is widely used in the food and cosmetic industries as a gelling, thickening, and stabilizing agent.
- K-Carrageenan forms a gel upon cooling or in the pres-ence of a gel-inducing reagent such as potassium chloride.

- Various cations, such as ammonium, calcium, alu-minum, and magnesium, also serve as good gel-inducing reagents.
- The conditions of immobilization by this method are mild.
- Another advantage of this method is that various shapes of the immobilized biocatalyst can be made.
- Disadvantage is the leaking of the biocatalyst can occur easily owing to the dissolution of the gel, when a gel-inducing reagent is not present in the reaction mixture.
- To increase the stability of the K-carrageenan-entrapped biocatalyst, treatment with glu-taraldehyde or hexamethylenediamine after entrapment is often effective.

Typical procedures for preparing various shapes of *K*-carrag-eenan gel (43) are as follows.

1. Cube Type

- The enzyme (100 mg) or microbial cells (16 g wet weight) are dissolved or suspended in 32 or 16 ml of physiological saline, respectively, at 25 to 50°C, and 3.4 g of carrageenan is dissolved in 68 ml of physiological saline at 37 to 60°C.
- The two are mixed, and the mixture is cooled at about l0°C for 30 min.
- To increase the gel strength, the gel is soaked in cold 0.3 M potassium chlonde solution. After this treatment, the resulting stiffer gel is cut into cubes (3 by 3 by 3 mm).

2. Bead Type

- A mixture (5 ml) of carrageenan and an enzyme or micro-bial cells is dropped into a 0.3 M potassium chloride so-lution through a nozzle having an orifice of 1 mm in diameter at a constant speed.
- Gel beads of 3 mm in di-ameter are obtained by this procedure.

3. Membrane Type

- A mixture (5 ml) of carrageenan and an enzyme or micro-bial cells is spread on a plate to form a thin layer (1 by 250 by 200 mm) and soaked in cold 0.3 M potassium chlo-ride solution to obtain a gel membrane.

C. Synthetic Resin Prepolymer Method

- With the application of the immobilized biocatalyst in a variety of bioreactions, including synthesis, transformation, degradation, or analysis, each having a different desirable chemical environment, finding a suitable gel among the natural polymers may be difficult.
- Synthetic resin pre-polymers such as photo-crosslinkable resin prepolymers and urethane prepolymers extend the list of polymers used for entrapment.
- In the synthetic resin prepolymer method the following properties of the entrapment gel can be regulated by the chain length of the prepolymer and he content of the reactive functional group
  a. Size of the gel matrix, which affects the substrate and product dif-fusion in the gel
  b. Mechanical strength of the gel formed
  c. Biocatalyst-holding capacity
  d. Growth capability of the cells inside the gel
- hydrophilicity or hydrophobicity of the gel can be controlled by selecting a suitable prepolymer synthesized in advance in the ab-sence of biocatalyst.
- The hydrophilicity-hydrophobicity balance of the gel is often critical in affectmg the partition of the reactant to the gel in a bioconversion reaction.
- The ionic properties of the gel, which are an important factor for a bioconversion reaction, can be introduced to the pre-polymers beforehand.

Advantages

i. En-trapment procedures are very simple and proceed under very mild conditions
ii. The prepolymers do not con-tain monomers that may have an unfavorable effect on the biocatalyst.

Therefore, the synthetic resin prepolymer method may be one of the most widely applicable immo-bilization methods at present.

1. Photo-Crosslinkable Resin Prepolymer Method

- The use of photo-crosslink able resin prepolymer that is hydrophilicor hydrophobic, cationic or anionic, and of different chain lengths has been developed.
- A mixture of the prepolymer and the biocat-alyst is gelled by irradiation with near-UV light for several minutes in the presence of a proper photosensitizer (initi-ator) such as benzoin ethyl ether.
- Photo-crosslinkable resin prepolymers can be autoclaved at 120°C.
- This method has been applied for the pilot-scale continuous production of etha-nol by immobilized, growing yeast cells

Method of Entrapment of Catalase with Photo-Crosslinkable Resin Prepolymer

- The photo-crosslinkable resin prepolymer (1 g) is mixed with 10 mg of an ini-tiator, benzoin ethyl ether, and 0.5 ml of 50 mM potassium phosphate buffer (pH 72) and melted by heating at 60°C.
- To the molten mixture is added 2.2 ml of the chilled buffer to cool the mixture and then 0.3 ml of the solution of bacterial catalase.
- The mixture is layered on a sheet of transparent polyester (7 by 10 cm) bounded by adhesive tape (thickness, 0.5 mm).
- The layer is covered with the same kind of sheet to eliminate exposure to the air and is illuminated by near-UV light (wavelength range, 300 to 400 nm; maximum intensity at 360 nm) for 3 min.
- The enzyme film thus formed (thickness, ca. 0.5 mm) is cut into small pieces (ca. 5 by 5 mm each) and used as the immobilized catalase.

2. Bead Type

- Ten parts (by weight) of photo-crosslinkable resin prepolymer are mixed with 0.08 parts of the initiator, 2

parts of 3% sodium alginate, 2 parts of distilled water, and 2 parts of centrifuged Zymomonas mobilis cells.

- This mixture is dropped into a 1.5% calcium chloride solution to form the beads.
- The initial cell concentration in this mixture is about 4 X 107 cells/ml.
- The beads thus formed are irra-diated by near-UV light with a wavelength of 300 to 400 nm.
- After irradiating for 5 min, the immobilized cell beads obtained are washed with sterile water.
- For practical application, the bead type is often preferred to the cube type or the film type.

3. Urethane Prepolymer Method

- There are many types of urethane pre-polymers having different hydrophilicity or hydrophobicity and chain length.
- The ure-thane prepolymers react with each other in the presence of water to form urea bonds and liberate carbon dioxide.
- Therefore, the entrapment can be carried out simply by mixing the prepolymer with an aqueous solution or suspension of the biocatalyst.
- The urethane pre polymers can be sterilized at 120°C by avoiding moisture.

Method of Entrapment of Invertase

- The urethane prepolymer (2.0 g), melted by incubation at 50°C (if necessary), is cooled to 4°C and mixed quickly and well with 2 ml of chilled 0.1 M phosphate buffer (pH 5.0) containing 20 mg of invertase in a small beaker.
- When gelation starts after a few minutes of mixing at room temperature, the mixture is kept at 4°C for 30 to 60 min to complete the polymer-ization.
- The resin gel thus formed is cut into small pieces (about 5 by 5 by 5 mm), washed thoroughly with the buffer, and used as immobilized invertase for the hydrolysis of sucrose.
- If the mixture, quickly and well stirred, is im-mediately layered on a glass plate framed with adhesive tape with the proper dimensions before the gelation starts, a film of the immobilized biocatalyst can be obtained.

Objectives

To compare the effectiveness of three methods of enzyme immobilization by gel entrapment.

Introduction

Three different commonly used entrapment media will be introduced in this experiment: polyacrylamide, calcium alginate, and gelatin. All these gels can be formed with a simple set of equipment and share similar procedures. In all the protocols, enzymes are well mixed with monomers/polymers and cross-linking agents in a solution. The solution is then exposed to polymerization promoters to start the process of gel formation. The solution is poured into a mold to achieve the desired shapes. A gel block may be cut into smaller cubes to increase the surface area. Commercially, it is common to force the unpolymerized solution through a set of nozzles to form spherical beads, whose size can be controlled by adjusting the back pressure. The resulting beads may be further hardened to enhance structural integrity.

Of the three gels, polyacrylamide is the most widely used matrix for entrapping enzymes. It has the advantage that it is non-ionic. The consequence is that the properties of the enzymes are only minimally modified in the presence of the gel matrix. At the same time, the diffusion of the charged substrate and products is not affected, neither. However, dimethylaminopropionitrile, the polymerization initiator, is highly toxic and must be handled with great care. The requirement to purge the monomer solution with nitrogen is also troublesome, although not totally crippling.

Calcium alginate is just as widely used as polyacrylamide. Unlike polyacrylamide gels, gelation of calcium alginate does not depend on the formation of more permanent covalent bonds between polymer chains. Rather, polymer molecules are cross-linked by calcium ions. Because of this, calcium alginate beads can be formed in extremely mild conditions, which ensure that enzyme activity yields of over 80% can be routinely achieved. However, just as easily as calcium ions can be exchanged for sodium ions, they can also be displaced by other ions. This property can both be advantageous and disadvantageous. If needed, enzymes or microbial cells can be easily recovered by dissolving the gel in a sodium solution. On the other hand, proper caution must be exercised to ensure that the substrate solution does not contain high concentrations of those ions that can disintegrate the gel.

The main attraction of using gelatin as the immobilization media is that the gel formation process requires only simple equipment and that the reagents are relatively inexpensive and nontoxic. The retention of enzymatic activities for immobilization with a gelatin gel is typically 25-50% of the original free enzyme. Gelatin gel has the advantage that the mass transfer resistance is relatively low compared to other entrapment methods, but the rate of enzyme loss due to leakage is high.

In summary, the efficiency of an immobilization process can be measured by the following criteria. Most important of all, a high percentage of the enzymes must be initially retained in gel matrices. Secondly, the enzyme activity must be preserved. And thirdly, the enzymes must be physically restrained from diffusing back into the substrate solution at a later time. It is quite difficult to create a fine and uniform mesh so as to prevent the entrapped enzymes from leaking out of the matrices. On the other hand, highly cross-linked matrices can result in higher mass transfer resistances for both the substrate and the product.

Bacterial alpha-amylase will be used in this experiment to demonstrate and compare the effectiveness of various entrapment techniques. The class will be divided into groups of three. Before attempting the experiment, identify the major steps and devise a work plan among the group members. Each member should be explicitly assigned an equal portion of the responsibilities. For example, one member may be responsible for making all three types of gels, and other members may be assigned the tasks of measuring the enzyme leakage and immobilized enzyme activities. Alternatively, each member may

be responsible for remain responsible throughout all phases of the study associated only one type of gel. It is the responsibility of the entire group to make sure that each member is carrying out the work according to the plan, whatever the plan is. Work closely together and help each other if needed to coordinate the smooth execution of the plan. Share the data at the end.

List of Reagents and Instruments

A. Equipment

- Erlenmeyer flasks
- Beakers
- Graduated cylinder
- Pipets
- Test tubes
- Temperature bath
- Thermometer
- Balance
- Syringe
- Spectrophotometer
- See Supplement A, Supplement B, and Supplement C for the equipment required in each immobilization protocols.

B. Reagents

- Bacterial alpha-amylase
- HCl solution, 1N
- KOH solution, 1N
- See Supplement A, Supplement B, and Supplement C for the reagents required in each immobilization protocols.

Procedures

1. *Prepare immobilized enzyme beads:* Immobilize alpha-amylase by entrapping it inside gel matrices according to the immobilization protocols accompanying this write-up. See Note 1.
2. *Immobilized enzyme activities:* Follow a similar procedure as in the previous amylase experiment to measure the activities of the immobilized enzymes. Instead of the enzyme solution, immerse about 1 g of the gel beads prepared in the above step in 10 ml of the buffered starch solution at pH=7.0. Constantly shake the mixture to make sure that the solution is not stagnant. As before, add 0.5 ml of the reacted starch solution to 5ml of the 0.1N HCl stopping solution. Mix with the iodine solution to detect the presence of residual starch. Rinse the gel beads with water and reuse the same beads twice more, each time measuring the enzyme activities.
3. *Shift in the optimal enzymatic condition — pH effect:* Study the effect of pH on the activities of the immobilized enzymes. Refer to the previous experiment on alpha-amylase. Because of the time limitation, perform this part for only one of the gels of your choice.
4. *Recovery from adverse pH condition:* Immerse about 1g of the gel bead in 5 ml of the 1N HCl solution. After shaking for 15 minutes, discard the HCl solution and thoroughly wash the gel beads with water. Measure the enzyme activity with buffered (pH=7.0) starch solution. Repeat for a 1N KOH solution.
5. *Recovery of enzymes:* Dissolve the gels and measure the enzyme activities afterward.
6. *Enzyme leakage and inactivation:* Immerse 5 ml of the fresh gel which has not been exposed to adverse pH or temperature conditions in 5 ml of water in a test tube for over 24 hours. Record the duration allowed for enzyme leakage. Measure and report the amylase activity in the surrounding water by following the same procedure as in the previous experiments. In addition measure the activities of the immobilized enzyme gel beads.
7. *For Curious Students:* Follow the same procedure as in the previous amylase experiment to study the effect of temperature on the activities of the immobilized enzymes. Is there any shift in the optimal temperature?

Notes

1. Note that each protocol produces approximately 10 ml of gels, barely enough for the explicitly stated steps of this experiment. However, if the student plans to perform additional investigation on his own, or if he feels that he is prone to repeated mistakes, double or triple the amount stated in the recipes so that there is a small reserve.

Discussions

Because enzymes are biological catalysts that promote the rate of reactions but are not themselves consumed in the reactions in which they participate, they may be used repeatedly for as long as they remain active. However, in most of the industrial, analytical, and clinical processes, enzymes are mixed in a solution with substrates and cannot be economically recovered after the exhaustion of the substrates. This single use is obviously quite wasteful when the cost of enzymes is considered. Thus, there is an incentive to use enzymes in an immobilized or insolubilized form so that they may be retained in a biochemical reactor to catalyze further the subsequent feed. The use of an immobilized enzyme makes it economically feasible to operate an enzymatic process in a continuous mode.

Numerous methods exist for enzyme immobilization, sometimes referred to as enzyme insolubilization. The overwhelming majority of the methods can be classified into four main categories: matrix entrapment, microencapsulation, adsorption, and covalent binding. Of these methods, matrix entrapment is the focus of this experiment.

Many entrapment methods are used today, and all are based on the physical occlusion of enzyme molecules within a “caged” gel structure such that the diffusion of enzyme molecules to the surrounding medium is severely limited, if not rendered totally impossible. What creates the “wires” of the cage is the cross-linking of polymers. A highly cross-linked gel has a fine “wire mesh” structure and can more effectively hold smaller enzymes in its cages. The degree of cross-linking depends on the condition at which polymerization is carried out. Because there is a statistical variation in the mesh size, some of the enzyme molecules gradually diffuse toward the outer shell of the gel and eventually leak into the surrounding medium. Thus, even in the absence of loss in the intrinsic enzyme activity, there is a

need to replenish continually the lost enzymes to compensate for the loss of apparent activity. In addition, because an immobilized enzyme preparation is used for a prolonged period of operation, there is also a gradual, but noticeable, decline in the intrinsic enzyme activity even for the best method. Eventually, the entire immobilized enzyme packing must be replaced.

Besides the leakage of enzymes, another problem associated with the entrapment method of immobilization is the mass transfer resistance to substrates, products, and inhibitors. Because the average diameter of a typical bead of enzyme impregnated gel is much larger compared to the average diffusion length, substrate cannot diffuse deep into the gel matrix, as in any other conventional non-biological immobilized catalysts. At the same time, the diffusional resistance encountered by the product molecules can sometimes cause the product to accumulate near the center of the gel to an undesirable high level, leading to product inhibition for some enzymes. Thus, ideally the network of cross-linking should be coarse enough so that the passage of substrate and product molecules in and out of a gel bead is as unhindered as possible. For this reason, entrapment is not suitable for special cases where the substrate has a large molecular weight such that it cannot easily move freely in the gel matrix.

Unlike the adsorption and covalent bonding methods, most polymerization reactions that cause cross-linking and gel formation in entrapment methods do not directly involve the formation of bonds between the support material and the enzyme molecules. There are reports that these bonds change the conformation of the enzyme protein and modify the enzyme properties. Since the enzyme molecules do not themselves participate in the polymerization reaction in the entrapment methods, the same entrapment techniques can be successfully applied to a wide range of enzymes with only minor modifications between different enzymes.

Questions

1. Compare the activities of the immobilized beads of different methods. Be sure that the comparison is based on the same standard and is fair.
2. How would you measure the mass transfer resistance in a gel matrix?
3. How would you estimate the relative contribution of the following factors in the overall loss of enzyme activities: enzyme deactivation, enzyme leakage, mass transfer?
4. How fast did the activity decline over a period of one or more days, if at all? What fraction of that decline can be attributed to the leakage of the enzyme from the gels?
5. Was there any shift in the optimal pH or temperature due to immobilization? Do you expect any?
6. What industrial enzymatic processes are routinely carried out with immobilized enzymes instead of free enzymes? What methods of immobilization are actually being used?
7. List some of the advantages and disadvantages of an immobilized enzymatic conversion process versus a free one.
8. Comment on ways to improve the experiment.

Method

This technique is based on the polymerization of acrylamide with N,N'-methylene-bis-acrylamide (Bis) as the cross-linking agent. The degree of cross-linking, thus, can be partly controlled by adjusting the ratio of acrylamide to Bis used.

List of Reagents and Instruments

A. Equipment

- Beakers
- Pipets
- Balance
- Graduated cylinder
- Syringe and needle

B. Reagents

- Buffered Monomer Solution
  - o 0.1 mM EDTA
  - o 0.1 M Tris-HCl
  - o 11 g/l N,N'-Methylene-bis-acrylamide $(CH_2=CHCONH)_2CH_2$
  - o 200 g/l Acrylamide $CH_2=CHCONH_2$
  - o Adjust the pH to 7.0 (See Note 1)
- Washing Solution
  - o 0.5 M NaCl
  - o 0.1 mM EDTA
  - o 0.1 M Tris-HCl
  - o Adjust the pH to 7.0
- Dimethylaminopropionitrile (polymerization initiator)
- Potassium persulphate solution, 10g/l (polymerization catalyst)
- Nitrogen gas cylinder
- Enzyme

Procedures

1. Buffered Monomer Solution: Add 1.1 g of Bis and 20 g of acrylamide to a 100 ml of buffered solution (pH 7.0) of 0.1mM EDTA and 0.1M Tris-HCl in a beaker. See Note 1.
2. To 10 ml of the buffered monomer solution of the above step, add enzyme powders (approximately 0.1ml of 75g/l fungal amylase) or an equivalent concentrated enzyme solution; mix.
3. For 20 minutes, purge the dissolved oxygen in the solution that can interfere with the polymerization process with nitrogen. This step is critical in achieving a high degree of cross-linking.
4. Add 0.1 ml of dimethylaminopropionitrile; mix.
5. Add 1.0 ml of freshly prepared 10g/l potassium persulphate solution to initiate polymerization.
6. Now is the time to pour the solution into a mold if one does not desire the gel to form in the original beaker. Leave the solution undisturbed; gel will form in approximately 10-30 minutes. (Hardening can be accelarated by using more dimethylaminopropionitrile.

7. Cut the resulting gel into small cubes of approximately 3mm per side. Alternatively, if smaller pieces are desired, the gel can be forced through a syringe fitted with a fine needle.
8. Gently wash the free enzyme off the gel surface in 10 ml of the Washing Solution. Repeat the washing process two additional times.

Notes

1. The pH of the buffer should be adjusted to match the optimum value of the enzyme to be entrapped.

Discussions

The above methods of enzyme immobilization by gel entrapment can be directly applied to live cells with minor modifications. For example, dimethylaminopropionitrile used in forming the polyacrylamide gel may not be employed because of its toxicity to viable cells. The monomers of acrylamide are also somewhat toxic to cells. On the other hand, cells can be immobilized with much less degree of cross-linking due to its much larger size.

The main advantages of immobilized enzymes are:

- Easy separation from reaction mixture, providing the ability to control reaction times and minimize the enzymes lost in the product.
- Re-use of enzymes for many reaction cycles, lowering the total production cost of enzyme mediated reactions.
- Ability of enzymes to replace multiple standard chemical steps and provide enatomerically pure products.

Methods of fixation

- Simple adsorption onto a hydrophobic resin.
- Adsorption onto a resin followed by glutaraldehyde crosslinking.
- Ionic bonding (with H^+ or OH^- form resins).
- Covalent bonding via $-NH_2$ groups.
- Via enzyme cofactors such as Fe, Ni, Al etc.

Notes