Review Article

Enzyme immobilization: the state of art in biotechnology

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Abstract

The advantages of immobilized enzyme over its soluble counterpart arise from their improved stability and easy separation from the reaction media, leading to decrease in production cost. Immobilization methods range from adsorption onto matrices, entrapment, cross-linking and covalent bonding to prefabricated carriers or activated supports. Changes in kinetic properties of immobilized enzyme can produce substrate or pH gradient, which reduce the reaction rates and finally product yields.

Keywords: *Enzyme, Substrate, Matrix, Immobilization.*

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INTRODUCTION

Enzymes are biomolecules synthesized by the cell in order to bring about certain biochemical reactions under mild conditions. Enzymes or biocatalysts act on substrates specifically and ultimately cause transformation/conversion of substrates to products. Biocatalysts are medicinally, industrially and commercially important biomolecules. The advantages of biocatalysts over inorganic catalysts include; high specificity, high rate of reaction, non-toxicity, water solubility, biodegradability reproducibility under normal laboratory conditions, mild conditions of pH, temperatures and pressure. This article attempts to review immobilization techniques employed in immobilized microbial enzymes, which is the state of art in biotechnology. There are many reasons to immobilize enzymes. In addition, to the convenient handling of enzyme preparation, the two main benefits are 1) easy separation of enzyme from product and 2) reuse of the enzyme. Easy separation of the enzyme from the product simplifies enzyme application and permits reliable and efficient reaction technology. Enzyme reuse provides a number of cost advantages that are often an essential prerequisite for establishing an economically viable enzyme catalyzed process. The properties of immobilized enzyme preparations can be governed by the properties of both the enzyme and the carrier materials. Characteristic parameters of enzymes and carriers are summarized in Table 1.

However, immobilized enzymes are currently the subject of considerable attention for their advantages over soluble enzymes and the steadily increasing number of applications for immobilized enzymes.

Enzyme Immobilization Methods

Enzymes can be immobilized by different techniques, but they are categorized as follows:

1. Adsorption onto an inert carrier.
2. Entrapment within the lattice of a polymerized gel (synthetic and non-synthetic).

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Table 1: Characteristic properties of enzymes and carriers.

Enzyme characteristics:	**Carriers characteristics:**
Molecular mass, prosthetic groups, functional groups present on the surface, purity, specific activity, pH, temperature profiles, kinetic parameters for activity, inhibition, stability against pH, temperature, solvent, contaminants and impurities.	large surface area, permeability, hydrophilic/hydrophobic characters, insolubility, chemical/mechanical stability, high rigidity, suitable shape and particle size. Resistance to microbial attack and regenerability.

3. Cross linking of the protein with a bifunctional reagent.

4.Covalent bonding to a reactive insoluble support.

Adsorption

physical adsorption of an enzyme onto a solid support is probably the simplest way of preparing immobilized enzymes. The method relies on non-specific physical interaction between the enzyme protein and the surface of the matrix brought about by mixing a concentrated solution of enzyme with the solid. Among advantage of adsorption as a general method of insolublizing enzyme is that usually no reagents and only minimum steps of activation are required, as a result adsorption is cheap, easily carried out, and tends to be less disruptive to the enzyme than by chemical means of immobilization, the binding occurs mainly by hydrogen bonds, multiple salt linkages and Van der Waal's forces. In this respect, the method bears the greatest similarity to the situation found in biological membrane *in vivo* and has been used to model such systems (Chibatta, 1978). Because of the weak bonds formation among enzyme and the carrier, the enzyme can be leaked out due to changes in temperatures, pH, ionic strength or even the mere presence of substrate (Daneils and Farmer, 1981). Another disadvantage is non-specific further adsorption of other proteins or other substances as the immobilized enzyme is used. This may alter the properties of the immobilized enzyme or, if the substance adsorbed is a substrate for the enzyme, the rate will probably decrease, depending on the surface mobility of enzyme and substrate. Stabilization of enzymes temporarily adsorbed onto a matrix has been achieved by cross linking the protein in a chemical reaction subsequent to its physical adsorption (Sankaran *et al.,* 1989). Some of the carriers used to immobilize enzymes by physical adsorption are: activated carbon, bentonite, kaoline, collagen, alumina, Amicon-AP10, Diatomaceous earth, silanized alumina, silica gel, calcium carbonate, titanium, propyl agarose, nitrocellulose fiber, cheese cloth. DEAE-Cellulose, TEAE-Cellulose, DEAE-Sephadex, CM-Sephadex, CM-Cellulose, Amberlite XE-97, polyamino polystyrene, DEAE-Sephadex A-50, Duloite S-761, ion exchange resin, Amberlite CG-50, Dowex 2-anion-exchanger, Dowex 50-cation exchanger (Taylor, 1991).

Entrapment within the lattice of a polymerized gel (synthetic/non-synthetic)

Confining enzymes within the lattices of polymerized gels is another method for immobilization (Dinelli *et al.*,1974). This allows the free diffusion of low molecular weight substrates and reaction products. The usual method is to polymerize the hydrophilic matrix in an aqueous solution of the enzyme and break up the polymeric mass to specific particle size as there is no bond formation between the enzyme and polymer matrix. Inclusion provides a generally applicable method in theory involves no disruption of the protein molecules. However, free radical generated on the course of the polymerization may affect the activity of entrapped enzymes.Another disadvantage is that only low molecular weight substrates can diffuse rapidly in the enzyme rendering the method unsuitable for enzymes that act on macromolecular substrates such as ribonuclease, trypsin,dextranase. The bond distribution in pore size of synthetic gels of the polyacrylamide type inevitably results in leakage of the entrapped enzyme,even after prolonged washing. This may be overcome by cross-linking the entrapped protein with glutaraldehyde (Sankaran *et al.,* 1989).

Cross-Linking

Immobilization of enzymes has been achieved by intermolecular cross-linking of the protein, either to other protein molecules/polymerized gel (synthetic and non-synthetic) or to functional groups on an insoluble support intra matrix. Cross-linking of enzyme to itself has also been achieved. In this case the protein material will invariably be acting as a support. Furthermore, an artificial cross link can be formed within a polypeptide chain (an intramolecular cross-link), such as internal cross-link can stabilize the protein by preventing unfolding under stress conditions. It is possible to link two polypeptides via an intermole-

cular cross-link (Ciaron, 2003). A particular form of this involves the cross-linking of small enzyme crystals with the bifunctional reagent. Since the enzyme is in a stable particulate form, it can easily be separated from the reaction mixture and reused. Cross-linked enzyme crystals (CLECs) are a novel form of immobilized enzymes designed for application in industrial biotransformation process (Brown *et al*., 1998; Zelinski and Waldmann, 1997; Lalonde, 1997; Lalondle, Novia *et al.*, 1997). Cross-linked enzyme crystals are a suitable preparation that confers structural resistance to proteins by stabilization of the crystalline matrix (Govardhan, 1999; Margolin, 1996). In crystals, protein molecules are symmetrically arranged and their native conformation is stabilized. These crystalline biocatalysts are more stable than soluble enzymes, when exposed to organic solvents and high temperatures; conditions normally found in many industrial process (Shenoy *et al*., 2001).

Covalent bonding to a reactive insoluble support

The immobilization of enzymes on solid supports by covalent coupling usually leads to very stable preparations with extended active life when compared with immobilized enzyme preparations obtained with other coupling methods. The advantage of enzyme immobilization by covalent bonding is based on strong covalent bonding, so that the stable immobilized enzyme preparations have been obtained, which do not lose protein in the solution, even in the presence of high ionic strength solution. The disadvantages includes selection of conditions for immobilization by covalent binding is more difficult than in other carrier binding methods. The reaction conditions required are some- what complicated. To achieve higher activities in the resulting immobilized enzyme preparations by preventing inactivation reactions with the essential amino acid residues of the active site, several attempts have been made: (1) covalent attachment of the enzymes in the presence of a competitive inhibitor or substrate; (2) a reversible covalently linked enzyme-inhibitor complex; (3) a chemically modified soluble enzyme whose covalent linkages to the matrix is achieved by newly incorporated residues; (4) a zymogen precursor.

The main factors taken into account for covalent immobilization of enzymes is that (i) the functional group of proteins is suitable for covalent binding under mild conditions (Taylor 1991). Table 2 shows the reactive residues of enzymes involved in covalent immobilization. (ii) the coupling reactions between the proteins and the supports. Table 3 shows characteristics of the protein supports and (iii) the functionalized supports suitable for protein immobilization.

Table 4 indicates some of functionalized supports employed for protein immobilization. The coupling of protein molecules to solid supports involved mild reactions between amino acid residues of the protein and several groups of functionalized carriers. The major classes of coupling reactions used for the immobilization of proteins are: diazotization, amido (peptide) bond formation, alkylation and arylation, Schiff's base formation, Ugi reaction, amidination, thiol disulfide interchange reactions, mercurry enzyme interaction, γ- irradiation as shown in Table 5 for Immobilization of proteins by covalent bonding.

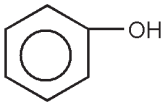
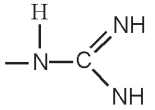
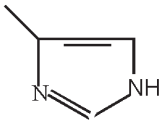
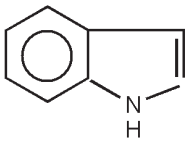
Choice of Immobilization Method

When immobilizing an enzyme on a surface it is most important to choose a method of attachment aimed to reactive groups outside the active catalytic and binding site of that enzyme. Considerable knowledge of active sites of particular enzymes will enable methods to be chosen that would avoid reaction with the essential group therein. Alternatively, these active sites can be protected during attachment as long as the protective groups can be removed without loss of enzyme activity. In some cases, this protective function can be fullfilled by a substrate of the enzyme or a competitive inhibitor;this also contributes towards retention of tertiary structure of the enzyme.

The surface on which the enzyme is immobilized has several vital roles to play such as retaining of tertiary structure in the enzyme by hydrogen bonding or the formation of electron transition of tertiary structure in the enzyme by hydrogen bonding or the formation of electron transition complexes. Retention of tertiary structure may also be a vital factor in maximizing thermal stability in the immobilized state. In this respect it is wise to follow closely the new findings in the chemical nature of soluble thermostable enzyme. The microenvironment of surface and the immobilized enzyme has an anionic or cationic nature of the surface that can cause a displacement at the optimum pH of the enzyme up to 2 pH units. This may be accompanied by a general broadening of the pH region in which the enzyme can work effectively.

Immobilization by cross-linking the protein enzyme in order to insolublize it or merely immobilize it in the desired location has many possibilities and is relatively cheap. Several aldehydes and other cross-linking agents are now available for this purpose. Extension of

Table 2. Reactive residues of proteins.

—NH_2	t-Amino of L-lysine (L-Lys) and N-terminus amino group
—SH	Thiol of L-cysteine (L-Cyc)
—COOH	Carboxyl of L-asparate (L-Asp) and L-glutamate (L-glu) and C-terminus carboxyl group
	Phenolic of L-tyrosine (L-Tyr)
	Guanidine of L-arginine (L-Arg)
	Imidiazole of L-histidine(L-His)
—S—S—	Disulfide of L-cystine
	Indole of tryptophan (L-Trp)
CH_3-S—	Thioether of L-methionine (L-Met)
—CH_2OH	Hydroxyl of L-serine (L-Ser) and L-threonine (L-Thr)

this approach to a process where an enzyme is an integral component of a copolymer could permit designing of reversible polymerization. Therefore, the immobilization method must be designed to minimize enzyme desorption, to maximize the stability of enzyme on the support, and to maximize the access of the substrate to the active site of the enzyme.

Glucoamylase produced by *Arthrobotrys amerospora* ATCC 34468 was adsorbed on to cheese cloth activated with polyethylneimine and then cross-linked with a bifunctional reagent. No shift in optimum pH of glucoamylase activity was observed as compared to the enzyme soluble form (Norouzian and Jaffar, 1993). Erarslan *et al*. (1990) studied the kinetic parameters of penicilline acylase immobilized on carboxymethyl cellulose (CMC). They found V_{max} and K_m to be 10.42 units and 1.02 mM for immobilized penicillin acylase and 10.53 units, 2.33 mM for soluble enzyme respectively. They conluded that adsorption on CMC had no effect on maximum velocity while K_m decreased more than two folds. This decrease in K_m was interesting for CMC penicillin acylase complex, because when the enzyme was covalently bound to aminochloro-s-

Table 3. Characteristics of the protein supports.

i-Large surface area.
ii-Permeability.
iii-Hydrophilic/hydrophobic characters.
iv-Insolubility.
v-Chemical, mechanical and thermal stability.
vi-High rigidity.
vii-Suitable shape and particle size.
viii-Resistance to microbial attack.
ix-Regenerability.

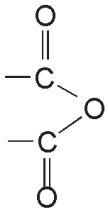
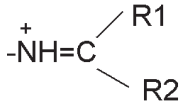
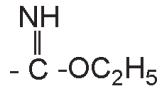
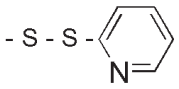
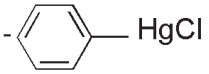
Table 4. Functionalized supports for protein immobilization.

Nucleophilic group of support	Activating reagent	Active derivative
-OH -OH	CNBr	O C=NH O
-OH -OH	$ClO_2C_2H_5$	O CH=O O
-OH	O=⟨⟩=O	O O O
-OH -OH	IO_4-	—COH —COH
$-NH_2$	Cl_2CS	—NCS
NH_2	Cl_2CS	NCS
NH_2	Cl_2CO	NCO
$-CONHNH_2$	$NaNO_2$	$—CON_3$
- COOH	R-N+C+N-R', H^+	—COOH
- COOH	CH_3OH, NH_2NH_2, HNO_2	$—CON_3$
	S—S	S—S

triazinyl DEAE-cellulose such a decrease could not be observed (Warburton *et al*., 1972). In entrapping the cells containing particular enzyme activity, diffusion of substrates in to the cells always remains obstacle. To overcome on this problem *E. coli* cells bearing penicillin acylase activity were first treated with N-acetyl-N,N,N,trimethylammonium bromide and then immobilized within porous polyacrylamide beads. The immobilized whole cells showed enhanced hydrolysis rates in the conversion of penicillin G to 6-aminopenicillanic acid compared to untreated cells entrapped and used under identical conditions. The immobilized system showed no apparent loss in enzyme activity when employed repeatedly over 90 cycles for 6-aminopenicillanic acid production (Prabhune *et al*., 1992). Likewise, Norouzian *et al*. (2002) permeabilized *E. coli* PPA78 cells bearing penicillin acylase activity with acetyltrimethyl-

ammonium bromide to entrap within the porous beads of gelatin and then cross-linked with glutaraldehyde. The permeabilization and entrapment within such a matrix reduced the diffusional restriction imposed by cell membrane, cell wall and avoided the generation of steep pH gradient across the column, due to formation of phenylacetic acid respectively. *Saccharomyces cerevisiae* PTCC5080 cells with alcohol dehydrogenase activity were also permeabilized with cetyltrimethyammonium bromide and then immobilized within the lattice of polyacrylamide beads. Permeabilization of the biocatalyst caused 27% increase in alcohol dehydrogenase activity. The immobilized system could be recycled 6 times, each cycle was of 6 h continuos operation (Norouzian *et al*., 2003). Different synthetic and non synthetic matrices

Table 5. Immobilization of proteins by covalent bonding.

Coupling reaction	Reacting group of protein	Reactive group of support
-O-CH_4-CH_2-SO_2-CH+CH_2	-NH_2 -SH -OH	Conjugation
	-NH_2	Peptide bond formation
-CHO Aldehyde		
	-NH_2	Schiff's base formation
 Imine	-CH_2H -NH_2	Ugi reaction
	-NH_2	Amidination
Imidoester		
	-SH	Thioldisulfide interchange
Disulfide residue		
	-SH	Mercury-enzyme interaction
Mercury derivative M' Matrix radical	E' Enzyme radical	γ-irradiated induced coupling

like polyacrylamide, alginate, gelatin were used to entrap tyrosinase of mushroom in order to produce L-3, 4-dihydroxyphenylalanine. The maximum immobilization yield of 88% was obtained for gelatin followed by 67 and 57% for Ca-alginate and polyacrylamide gels respectively (Manjal and Sawhny, 2002). Boadi and Neufled (2001) employed various biological matrices like, alginate, chitosan, carragenein, and pectin to immobilize tannase for hydrolyzing tannins present in tea. They could increase the retention of tannase in alginate gels by coating the alginate with high and low molecular weight chitosan and then cross-linked with glutaraldehyde. Entrapment of the enzyme in hollow fiber could be of interest when the substrate to be acted upon, is of low molecular weight. Naringinase, is an enzyme composed of two components namely, α-rhamnosidase and β-glucosidase, can be employed to debitter grapefruit juice. Tsen *et al.* (1991) entrapped naringinase of *Penicillium* species in hallow fibers of cellulose triacetate. The immobilized system could effectively debitter grapefruit juice under experimental conditions. Tsen, Tsai and Gee-Kaite (1989) also investigated the properties of naringinase entrapped in cellulose triacetate fiber and compared them with its soluble counter part. The optimal conditions like pH and temperature were similar to those of soluble enzyme but the immobilized enzyme showed higher k_m value as compared to soluble naringinase. Puri *et al.* (1996) studied the immobilization effect on naringinase of *Penicillium* species. The enzyme was entrapped in 2% Na-alginate beads so as to debitter kinow juice (*Citrus nobilis* and *Citrus deliciosa*). It was observed that the optimum pH of immobilized naringinase activity was broadened. This attributed flexibility for debittering of kinow (orange) juice at various pH values and temperature profiles revealed improved thermal stability. Tyrosinase was immobilized on nylon 6, 6 membrane using glutaraldehyde as a cross linking agent. Pialis *et al*., (1996) studied the effect of glutaraldehyde concentration in accordance with the membrane pore size upon enzyme uptake and L-3, 4-dihydroxyphenylalanine (L-DOPA) production. Excess of glutaraldehyde and larger pore size of membrane affected L-DOPA formation. The authors concluded that tyrosinase could be effectively immobilized on nylon 6, 6 and L-DOPA production was optimal when 0.2 μm pore size membrane were activated with 3-5% glutaraldehyde solution. L-3,4-Dihydroxy-phenylalanine was produced by immobilized tyrosinase. The enzyme was immobilized on sodium and calcium aluminosilicate and then cross-linked with glutaraldehyde. The behavior of the immobilized tyrosinase and strength of the enzyme attachment were studied. There was no loss of enzyme activity of the immobilized enzyme during 40-48 h of repeated batch operation. It was found that the stability of tyrosinase on these supports was much superior to the stability of the enzyme when immobilized on other supports (Gayathri *et al.,* 2002; Jimenez and Saville, 1996; Pialis *et al.,* 1996; Vilanova *et al.,* 1984). Lee *et al.* (2002) investigated the mechanical stability of cross-linked micro crystals of alcohol dehydrogenate in stirred tank reactor under agitation. The investigators could carefully control the crystallization condition and cross-linked the crystal with glutaraldehyde in order to form cross-linked enzyme crystals (CLECs). Cross-linking of enzyme crystals yields an immobilized biocatalyst with exceptional stability. The high purity (crystalline enzyme) together with the stability provided ideal pre-requisite for further modification of the active site by chemical methods resulting in a new catalytic activity. Henceforth, Dietmar and Schreier (1998) modified the active site and polypeptide frame of crystalline serine proteinase that yielded an active site modified enzyme with new catalytic properties called semi-synthetic peroxidase seleno-subtilisin. Cross-linking of the crystals with glutaraldehyde made the enzyme insoluble in water as well as organic media. Tuchsen and Ottesen (1997) described this phenomenon as during the reaction time, three lysine residues at the subtilisin surface had reacted with its neighbors in the crystal lattice. Protein crystals are porous materials in which the diffusion of small molecules is possible. To avoid mass transfer limitation micro-crystals less than 100 μm in one dimension are preferred. Therefore, chloroperoxidase of *Caldariomyces fumago* CMI89362 was crystallized and microcrystals retained their catalytic activity. The microcrystals were then cross-linked with glutaraldehyde which yielded water insoluble crystals (Ayala, 2002). This may be due to the ability of glutaraldehyde to form different sized polymers in solution, with the advantage that the distance between two amino acids could be covered by one of those species (Walt and Agayn, 1994). Supports such as macroporous weak cation exchanger methacrylate polymers were used to immobilize penicillin G acylase. The role of certain factors like pore generating solvents, cross- linking agents and comonomer in enzyme adsorption and expression were studied. Kerosin, ethylene glycol, dimethyl acrylate, acrylic acid and glutaraldehyde were served

as superior pore generating solvent, density causing agent, comonomer and cross-linking agent respectively. Therefore, the properties of penicillin G acylase immobilized on acrylic acid (copolymer PM-39) by adsorption and cross linking with glutaraldehyde (IME-PM-39) were studied. The optimal pH, temperature and K_m values of immobilized penicillin G acylase shifted from 8.0 to 7.5-7.8, 50ºC to 55ºC and 0.03 mol dm^{-3} to 2.4-3 mol dm^{-3} respectively as a result of immobilization on PM-39 (Koilpillai *et al.*, 1990). When naringinase was covalently linked to glycophase coated, controlled pore glass, no shift in optimum pH of the immobilized enzyme was observed but the energy of activation of the immobilized enzyme was decreased noticeably (from 14.9 to 7.9 Kcal/mol). This allowed a higher catalytic efficiency of immobilized naringinase. It was also observed that the immobilized enzyme was more stable in increasing ionic strength than soluble naringinase. Interestingly, immobilized enzyme derivative retained 85-90% of their α-rhamnosidase activity for a year when stored at 3-5ºC suspended in potassium hydrogen phthalate/HCl buffer pH 3.5 (Manjan *et al.*, 1985). Ellenrieder and Daz (1996) employed collagen, keratin and Fibrion to immobilize naringinase to debitter grapefruit juice, by activating the supports by 2-5% glutaraldehyde solution.They found that Fibrion of *Bombayx mori* silk was the most superior supports giving the greatest enzyme stabilization. Many investigators have studied the efficacy of covalent attachment of the enzyme on to different matrices. It is concluded that each support has its own advantages and disadvantages in accordance with the enzyme employed (Golstein, Lifshitz and Skolvoski, 1971, Ono *et al.,* 1978; Tsen, 1984). Naringinase produced by *Penicillium decombens* was immobilized through covalent attachment to seeds of *Ocimum basilicum* (Melo *et al,* 1986) via ethylenediamine arms established on the seeds. The immobilization of the enzyme on such a particular support was more effective where 57% of the total enzyme activity and 92% of the protein were attached to the activated seeds (Norouzian *et al.,* 1999). Penicillin V acylase produced by *Streptomyces lavendulae* was immobilized by covalent bonding onto Eupergit C. The immobilized enzyme showed an optimal pH values of 9.5-10.5 and temperature of 60ºC, whereas its soluble counterpart showed the same optimum pH value and lower temperature (50ºC) for penicillin V acylase activity (Torres *et al*., 2000).

CONCLUSION

The immobilization of enzymes is a useful tool to meet cost targets and has a number of technological advantages, as for instance, it enables repeated use of enzyme and hence produces significant cost savings and immobilized enzyme can easily be separated from the reaction liquid and thereby reduce laborious separation steps. Additional benefits arise from stabilization against harsh reaction condition, which are deleterious to soluble enzyme preparation .

Cross-linked enzyme crystals (CLECs) is a method of immobilizing enzyme, i.e. converting soluble form to insoluble counter part. Henceforth, CLEC is a new technique and very promising type of biocatalyst for industrial application. Moreover, new nano-materials will be produced by engineering specific covalent linkages into protein or enzyme crystals .

Strategic modifications in the surface of the protein via site-directed mutagenisis, followed by cross-linking of the crystals of these mutants along the crystallographic planes will produce sheets or fibers upon subsequent dissociation of the crystal. It is intended to exploit this new class of nano-materials for application such as ultrafiltration membrane, enzyme crystal morphologies and ultimately microelectronic devices. It promises the stability of three dimensional cross-linked enzyme crystals without the same substrate and product mass transfer resistances.

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