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Immobilization of Enzymes: Methods and Applications

Traditionally, enzymes in free solutions (i.e. in soluble or free form) react with substrates to result in products. Such use of enzymes is wasteful, particularly for industrial purposes, since enzymes are not stable, and they cannot be recovered for reuse.

Immobilization of enzymes (or cells) refers to the technique of confining/anchoring the enzymes (or cells) in or on an inert support for their stability and functional reuse. By employing this technique, enzymes are made more efficient and cost-effective for their industrial use. Some workers regard immobilization as a goose with a golden egg in enzyme technology. Immobilized enzymes retain their structural conformation necessary for catalysis.

There are several advantages of immobilized enzymes:

1. Stable and more efficient in function.
2. Can be reused again and again.
3. Products are enzyme-free.
4. Ideal for multi-enzyme reaction systems.
5. Control of enzyme function is easy.
6. Suitable for industrial and medical use.
7. Minimize effluent disposal problems.
8. high enzyme substrate ratio.
9. Minimum reaction time.
10. Continuous use of enzyme.

There are however, certain disadvantages also associated with immobilization.

1. The possibility of loss of biological activity of an enzyme during immobilization or while it is in use.
2. Immobilization is an expensive affair often requiring sophisticated equipment.
3. Some enzyme become unstable after immobilisation.
4. Sometimes enzymes become inactivated by the heat generated by the system.

Methods of Immobilization:

Adsorption: Adsorption involves the physical binding of enzymes (or cells) on the surface of an inert support. The support materials may be inorganic (e.g. alumina, silica gel, calcium phosphate gel, glass) or organic (starch, carboxymethyl cellulose, DEAE-cellulose, DEAE-sephadex).

Adsorption of enzyme molecules (on the inert support) involves weak forces such as van der Waals forces and hydrogen bonds. Therefore, the adsorbed enzymes can be easily removed by minor changes in pH, ionic strength or temperature. This is a disadvantage for industrial use of enzymes.

Entrapment: Enzymes can be immobilized by physical entrapment inside a polymer or a gel matrix. The size of the matrix pores is such that the enzyme is retained while the substrate and product molecules pass through. In this technique, commonly referred to as lattice entrapment, the enzyme (or cell) is not subjected to strong binding forces and structural distortions.

Some deactivation may however, occur during immobilization process due to changes in pH or temperature or addition of solvents. The matrices used for entrapping of enzymes include polyacrylamide gel, collagen, gelatin, starch, cellulose, silicone and rubber. Enzymes can be entrapped by several ways.

Microencapsulation: Microencapsulation is a type of entrapment. It refers to the process of spherical particle formation wherein a liquid or suspension is enclosed in a semipermeable membrane. The membrane may be polymeric, lipoidal, lipoprotein-based or non-ionic in nature. There are three distinct ways of microencapsulation.

1. Building of special membrane reactors.

2. Formation of emulsions.

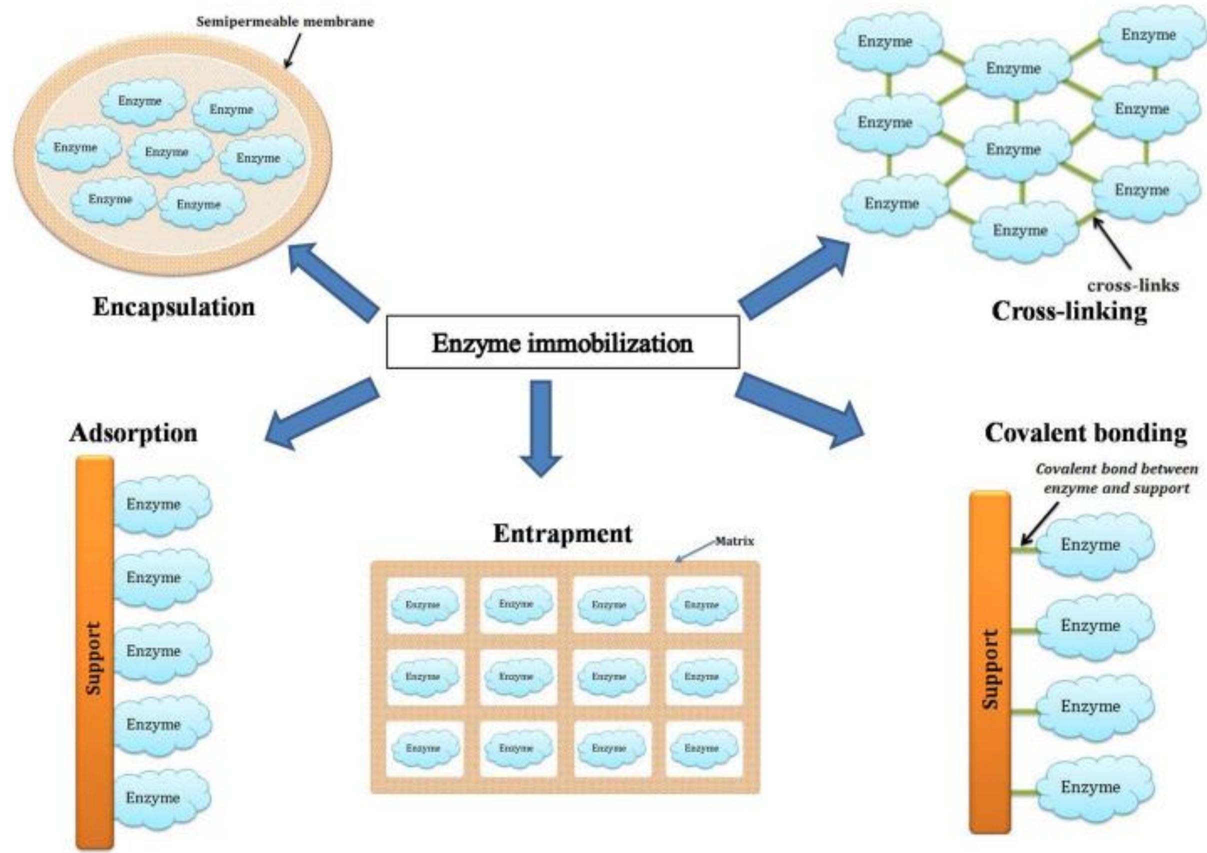
3. Stabilization of emulsions to form microcapsules.

Microencapsulation is recently being used for immobilization of enzymes and mammalian cells. For instance, pancreatic cells grown in cultures can be immobilized by microencapsulation. Hybridoma cells have also been immobilized successfully by this technique.

Covalent Binding: Immobilization of the enzymes can be achieved by creation of covalent bonds between the chemical groups of enzymes and the chemical groups of the support. This technique is widely used. However, covalent binding is often associated with loss of some enzyme activity. The inert support usually requires pretreatment (to form pre-activated support) before it binds to enzyme. The following are the common methods of covalent binding.

Cross-Linking: The absence of a solid support is a characteristic feature of immobilization of enzymes by cross- linking. The enzyme molecules are immobilized by creating cross-links between them, through the involvement of poly-functional reagents. These reagents in fact react with the enzyme molecules and create bridges which form the backbone to hold enzyme molecules. There are several reagents in use for cross-linking. These include glutaraldehyde, diazobenzidine, hexamethylene diisocyanate and toluene di- isothiocyanate.

Glutaraldehyde is the most extensively used cross-linking reagent. It reacts with lysyl residues of the enzymes and forms a Schiff's base. The cross links formed between the enzyme and glutaraldehyde are irreversible and can withstand extreme pH and temperature. Glutaraldehyde cross- linking has been successfully used to immobilize several industrial enzymes e.g. glucose isomerase, penicillin amidase. The technique of cross-linking is quite simple and cost-effective. But the disadvantage is that it involves the risk of denaturation of the enzyme by the poly-functional reagent.



Applications of enzyme immobilization:

(1). Industrial production: Industrial production of antibiotics, beverages, amino acids etc. uses immobilized enzymes or whole cells.

(2). Biomedical applications: Immobilized enzymes are widely used in the diagnosis and treatment of many diseases. Immobilized enzymes can be used to overcome inborn metabolic disorders by the supply of immobilized enzymes. Immobilization techniques are effectively used in drug delivery systems especially to oncogenic sites.

(3). Food industry: Enzymes like pectinases and cellulases immobilized on suitable carriers are successfully used in the production of jams, jellies and syrups from fruits and vegetables.

(4). Research: A Research activity extensively uses many enzymes. The use of immobilized enzyme allow researcher to increase the efficiency of different enzymes such as Horse Radish Peroxidase (HRP) in blotting experiments and different Proteases for cell or organelle lysis.

(5). Production of bio-diesel from vegetable oils.

(6). Waste water management: treatment of sewage and industrial effluents.

(7). Textile industry: scouring, bio-polishing and desizing of fabrics.

(8). Detergent industry: immobilization of lipase enzyme for effective dirt removal from cloths.

Immobilization of Glucose Isomerase

One of the ways to reduce the cost of production of GI is to recover it efficiently and reuse it several times. Immobilization of GI offers an excellent opportunity for its effective reuse. The largest market for GI is for its immobilized form. Development of immobilized GI has been a subject of great interest. The use of GI is expensive because it is an intracellular enzyme and large quantities are needed to compensate for the high Km for glucose. Therefore, it is important to immobilize GI for its industrial applications. Several methods for immobilizing GI have been described. However, only a few are economical and yield enzyme preparations with properties that are suitable for commercial production of HFCS. Two main methods are used for immobilization of GI: cell-free enzyme immobilization and whole-cell immobilization.

Cell-free immobilization: Soluble enzymes that are immobilized to a support structure have excellent flow characteristics suitable for continuous operations, in contrast to whole-cell immobilized supports, and offer considerable savings in terms of capital equipment. GIs from *Streptomyces phaeochromogenes* and *Lactobacillus breviswere* immobilized on DEAE-cellulose. The Streptomyces GI immobilized on DEAE-cellulose is being used to produce HFCS in a semi continuous plant by the Clinton Corn Processing Company. A GI preparation from *Streptomyces* sp.

Whole-cell immobilization: Because GI is an intracellular enzyme, whole-cell immobilization is the method of choice foremost of the commercially available

immobilized GIs. Whole cells containing GI were spray-dried and used in the first industrial process to produce HFCS by the Clinton Corn Pro-cessing Company. Addition of inorganic salts such as magnesium hydroxide to the fermentation broths of *Streptomyces* or *Arthrobacter* species followed by filtration and drying of the cake provided a straightforward method to immobilize cells containing GI. Physical entrapment of whole cells in polymeric materials was used as an immobilization method by Novo Industries, whereas chemical entrapment of cells in a membrane followed by cross-linking with glutaraldehyde was used to prepare an immobilized GI to be used on a commercial scale.

Production of High-Fructose Corn Syrup: The market development in HFCS production was marked by a gradual acceptance of HFCS and of the enriched HFCS (55% fructose) as substitutes for sucrose by soft-drink producers. The most common raw material used for the production of HFCS in the United States is the corn-starch manufactured by the wet milling process. The production of HFCS from starch comprises three major processes: (i) liquefaction of starch byaamylase, (ii) saccharification of starch by the combined action of amylo glucosidase and a debranching enzyme, and (iii)isomerization of glucose by GI. The final product is a corn syrup containing a mixture of glucose and fructose and hence with a greater sweetening capacity than that of sucrose. Other sources of starch such as wheat, tapioca, and rice are used to a minor extent in other parts of the world.

Advantages of HFCS as sweetener ¬ 1.3 times sweeter than sucrose and 1.7 times sweeter than glucose (Bhosale et al. 1996) ¬ Flavour enhancement ¬ Cost effective ¬ High stability ¬ Low water activity ¬ Resist crystallisation ¬ fermentable ¬ Diabetic sweetener ¬ Beverage, soft drinks, dairy products, baked food.

Production of Ethanol: GI catalyses the isomerization of both glucose and xylose. This property of the enzyme is used in the isomerization of xylose to xylulose, which can be ultimately fermented to ethanol by conventional yeasts. Bioconversion of renewable bio-mass to fermentable sugars and ethanol is important in view of the rapid depletion of fossil fuels. The biomass consists of cellulose (40%), hemicellulose (30%), and lignin (30%). The economic feasibility of biomass utilization depends on the hydrolysis of cellulose and hemicellulose to glucose and xylose and their subsequent fermentation to ethanol by yeasts. Until recently, the research efforts were focused on the bioconversion of cellulose. Then the awareness that the efficiency of bioconversion of lignocelluloses and agricultural wastes relied mainly on the effective utilization of the hemicellulose component of biomass shifted worldwide attention to hemicellulose fermentation. Xylan is a major constituent of hemicellulose and consists of xylose units linked by αβ(1,4) linkage. D-Xylose is easily produced by acid or enzymatic hydrolysis of xylan. Industrial yeast strains such as *Saccharomyces cerevisiae* generally ferment hexoses efficiently but D-xylose remains un-utilized. A few yeasts such as *Pachysolen tannophilus, Pichiastipitis, Candida utilis*, and *Candida shehataeare* known toutilize pentoses through the oxido-reductive pathway, but the rates of fermentation are very low. Moreover, their low ethanol tolerance and catabolism of ethanol in the presence of oxygen limit their commercial application. GI has been used to produce xylulose from xylose, which

otherwise represents a major metabolic block in the process of fermentation of xylose to ethanol by conventional yeasts such as *Saccharomyces cerevisiae, Schizosaccharomyces pombe,* and *Candida tropicalis*. Although fermentation rates and product yields for ethanol production from D-xylose are significantly lower than from D-glucose, technology is now emerging to improve the process by transferring the GI gene to the yeast and conducting the isomerization and fermentation of xylose to ethanol simultaneously.

GI-producing organisms

Species
Actinomyces olivocinereus, A. phaeochromogenes
Actinoplanes missouriensis
Aerobacter aerogenes, A. cloacae, A. levanicum
Arthrobacter spp.
Bacillus stearothermophilus, B. megabacterium, B. coagulans
Bifidobacterium spp.
Brevibacterium incertum, B. pentosoaminoacidicum
Chainia spp.
Corynebacterium spp.
Cortobacterium helvolum
Escherichia freundii, E. intermedia, E. coli
Flavobacterium arborescens, F. devorans
Lactobacillus brevis, L. buchneri, L. fermenti, L. mannitopoeus, L. gayonii, L. fermenti, L. plantarum, L. lycopersici, L. pentosus
Leuconostoc mesenteroides
Microbispora rosea
Microellobosporia flavea
Micromonospora coerula
Mycobacterium spp.
Nocardia asteroides, N. corallia, N. dassonvillei
Paracolobacterium aerogenoides
Pseudonocardia spp.
Pseudomonas hydrophila
Sarcina spp.
Staphylococcus bibila, S. flavovirens, S. echinatus

Commercial producers of GI

Organism	Trade name	Manufacturer
Actinoplanes missousriensis	Maxazyme	Gist Brocades and Anheuser-Busch Inc.
Bacillus coagulans	Sweetzyme	Novo-Nordisk
Streptomyces rubiginosus	Optisweet	Miles Kali-Chemie
	Spezyme	Finnsugar
Streptomyces phaeochromogenes	Swetase	Nagase
Arthrobacter sp.		Reynolds Tobacco
Streptomyces olivaceus		Miles Laboratories Inc.

Collected from **Molecular and Industrial Aspects of Glucose Isomerase SNEHALATA H. BHOSALE, MALA B. RAO, AND VASANTI V. DESHPANDE*

USES OF PENICILLIN G ACYLASE(PGA)

Industrial uses of PGA The most widespread use of PGAs is in the production of 6-APA from both Pen G and Pen V. Immobilized PGA enzymes mainly *from E. coli, B. megaterium* and *A. faecalisare* available from a number of commercial suppliers. Reactions are carried out at >5,000L scale under controlled conditions, the pH being either controlled at approximately 8.0, or slowly ramped from 7.0 to 8.5, depending upon the catalyst, as high as 8.5. Exposure to high temperature (>30º C) and pH (>8.0) is minimized to reduce inactivation of the enzyme and retain high product yield of the otherwise relatively unstable 6-APA. The use of PGA in large scale production of semi synthetic penicillins and cephalosporins is also widespread. These processes are focused on the condensation of an appropriate D-amino acid derivative with a β-lactam nucleus in a PGA catalysed reaction. This involves the direct acylation of nucleophiles such as 6-APA or 7-ADCA with free acids at low (<7.0) pH.

Production of 6-APA by immobilized PGA: Enzymatic production from benzyl penicillin (Pen G) of 6-aminopenicillanic acid (6-APA) represents one of the few commercially established enzymatic processes in the pharmaceutical industry. The enzyme employed, penicillin G acylase (PGA), is immobilized on various solid supports using conventional immobilization techniques. The performance of immobilized penicillin G acylase (IMPGA) is determined by the type of reactor used.This Enzyme is the starting material for the manufacture of penicillin derivatives, which are the most widely used β-lactam antibiotics. Both natural and semi-synthetic penicillins contain 6-aminopenicillanic acid. Different penicillin types differ in their attached side chains. Semi-synthetic penicillin may be produced by enzymatic removal at the side chain of native penicillins with subsequent attachment of a novel side chain to the resultant 6-aminopenicillanic acid core. Kinetically controlled synthesis involves an acyl group transfer reaction in which activated acids, esters or amides are used as the acylating agents. The yield of this type of reaction is dependent upon three different reactions carried out by the enzyme: 1) the synthesis of the β-lactam compound, 2) the hydrolysis of the activated acyl donor, and 3) the hydrolysis of the product. There are many ways to optimize such a reaction including optimizing pH, addition of suitable solvents and the use of high concentrations of acyl donor and nucleus. Using such controlled strategies, a number of different antibiotics are produced including amongst others the high volume antibiotic products amoxicillin, ampicillin, cephalexin 38and cefazolin39.An alternative use of PGA is in peptide synthesis. The acylase can be used for the protection and deprotection of amino groups of amino acids by direct enzymatic synthesis and acyl group transfer reactions. For example, PGA has been used as a biocatalyst in the synthesis of the sweetener aspartame, and further use has been in the preparation of D-phenyl dipeptides whose esters readily undergo ring closure to the corresponding diketopiperazines. Such peptides are used as food additives and as synthons for fungicidal, antiviral and anti-allergenic compounds. In addition, PGA can hydrolyse phenyl acetyl derivatives of a number of peptides and resolve enantiomers of some organic compounds. The commercial viability of any enzyme depends on its operational stability and reusability. Enzymes in free form are thermolabile and cannot be reused,

owing to their loss during downstream processing and purification of the product. Immobilization is the most important technique for stabilizing enzyme activity and enhancing its operational life. Immobilization does not necessarily enhance the enzyme’s stability, but this can be achieved by different modes of the immobilization matrix system. PGA is one of the most common commercially significant examples of enzyme reusability.

Collected from: PRODUCTION, IMMOBILIZATION AND INDUSTRIAL USES OF PENICILLIN G ACYLASE

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