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Immobilized Enzyme Technology: Potentiality and Prospects

Review Article

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Abstract

Enzymes are biocatalysts that catalyze a wide array of reactions. Enzyme immobilization is a technique where an enzyme is fixed to a support (more recently nanostructures) while retaining its catalytic activity. Natural or artificial substrates can be used as efficient carriers. A variety of both reversible and irreversible immobilization methods are available, viz., adsorption, chelation, affinity binding, covalent binding, cross linking, entrapment, encapsulation, etc. Each method has its own set of advantages and disadvantages. Hence, new or improved techniques of immobilization are being investigated. Immobilized enzymes have shown greater stability, high activity (comparable or even greater than free enzymes) and better tolerance to unfavourable conditions. This technology also prevents the loss of enzymes into the reaction medium (leaking of enzyme). Immobilized enzymes find applications in a wide array of fields, including food and textile industries, medicine, biodiesel production and other industrial sectors. Treatment of several diseases and bioremediation programs to remove toxic pollutants from the environment has also been undertaken using this technique. The potential of multi-enzyme immobilized systems as a tool to catalyze multi-step reactions are the present focus of the scientific community to ensure better industrial yields.

Introduction

Natural resources are being rapidly depleted due to the increasing ecological footprint. The demand for greater output and efficiency in industries as well as in the field of medicine is on the rise. Hence, it has become quintessential to practically use 'white biotechnology' to improve the efficiency of industrial processes through molecular biology tools including genetic engineering, in order to satisfy the growing demands of the population [1]. Enzymes or biocatalysts are being increasingly used nowadays in medicine for making novel drugs and detection of diseases, as well as in industries for cheese production, sugar and syrup production, meat tenderizer, food additive, fat and oil industry, textile industry, detergent industry, etc. [2]. The biochemical basis of synthesis results in better production, owing to much greater efficiency and far less detrimental contaminants, as compared to classical chemical synthesis [3]. However, the enzymes being biomolecules, catalysing reactions under optimum cellular conditions, often show unfavourable characteristics. These can be thermal instability, inhibition of activity and being prone to protease degradation. The enzymes are also significantly expensive and loss of enzymes in the purification step in industries leads to significant increase in capital investments [4]. Enzyme immobilization promisingly counteracts these drawbacks. The term immobilized enzymes refers to physical confinement or localization of enzymes within a defined matrix or support for retention of their catalytic activities so that they can be used repeatedly and continuously [5]. The ability to immobilize, re-use and purify an enzyme has great advantages, as it steadily reduces industrial investments and increases profit by making the entire process economically feasible [6]. Enzyme immobilization has been in the fray since early twentieth century.

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In 1916, Nielsen and Griffin found that invertase, bound to artificial substrate of aluminium hydroxide [$Al(OH)_3$] and charcoal, had retained its catalytic activity. However, the idea of exploiting these immobilized enzymes began only from 1950s, and by 1970s, the technology was quite developed [6]. Enzyme immobilization can be achieved by several methods, viz., adsorption, covalent binding, entrapment and encapsulation, affinity binding, chelation, etc. to name a few. Extensive research is being carried out to develop a multi-enzyme immobilization technology to facilitate better product yields in reactions involving multi-enzyme pathways.

Immobilization Techniques

Any immobilized enzyme contains two essential functions; (i) Non-Catalytic Functions (NCFs) that are designed to aid the separation of enzyme from the reaction mixture and hence plays a role in its re-use, (ii) Catalytic Functions (CFs) that are designed to convert the substrates into the products within the desired time and space. NCF relates to the physical properties of the immobilized enzyme, such as shape, size and length of the selected carrier. CF is correlated with the biological activities of the enzyme such as substrate specificity, activity, ideal pH and temperature range. Thus, it is mandatory to develop a 'robust' enzyme system which consists of an optimum NCF and a high CF to ensure purification and re-use, along with high product yield within a minimum time frame. The CFs are designed according to the reaction to be catalysed and the substrate involved, allowing fast and maximum product formation with negligible side reactions. The NCFs are designed on the basis of the nature of the bioreactor, the reaction medium and the methods of purification of product [6-9]. Hence, based on the requirements of the industry, both the carrier and the method of immobilization are chosen. A few methods of enzyme immobilization will be discussed in this review. These methods have been classified into two major groups [10] as follows:

Reversible Enzyme Immobilization

As the name suggests, this method is reversible, and the enzymes can be removed from the support easily by simple reactions or reversal of the conditions by which the immobilization was carried out. The method can be of the following types:

Adsorption

Adsorption (Figure 1a) is the oldest and arguably the simplest of all techniques. The first industrial process that involved immobilized enzyme used an aminoacylase adsorbed to a DEAE-Sephadex for continuous resolution of amino acids. The first record of large-scale industrial utilization of immobilized enzyme technology also involved adsorption of glucose isomerase to DEAE-Cellulose in the production of high fructose corn syrup by Clinton Corn Products [11].

Principle: This involves adhering of the enzyme to the surface of the carrier via several weak non-covalent interactions such as hydrogen bond, Van Der Waal's interactions and hydrophobic interaction [12].

Advantage: This method is cheap, easy to perform and allows easy recovery of the enzyme from the carrier, thus allowing re-use of both. So, it requires very little activation and no reagents [13]. The weak interactions involved, hardly cause any distortion of the enzyme retaining maximum enzyme activity [11]. A variety of organic and inorganic materials can be used as support. If the adsorption is based on hydrophobic interactions, it is stabilized by high ionic concentrations, thereby permitting the usage of high concentrations of substrate in the bioreactor [8].

Disadvantage: Significant enzyme loss cannot be avoided in this technique as the binding forces are weak, reversible and susceptible to physical parameters such as pH and temperature [13]. This may lead to the presence of enzyme in the reaction product, which can contaminate the products and complicate the purification process [11].

Carriers used: Common carriers used in adsorption are activated charcoal, alumina, cellulose, Sephadex, agarose, collagen and starch [13]. Researchers have come up with new eco-friendly carriers, such as coconut fibre with high water retention and cation exchange properties, which could significantly reduce costs as well as prevent ethical issues [14].

Chelation

Chelation or metal binding (Figure 1b) is another common type of reversible enzyme immobilization mostly used as a chromatographic method. Being reasonably expensive and involving safety issues, this method is less popular in industries.

Principle: It is based on the ability of charged and polar amino acids (histidine, lysine, phenylalanine, cysteine and tyrosine) to bind to metal ions via coordinate bonds. The metal ions bound to the carrier surface have metal ligands weakly bound to them. Upon exposure of the enzyme to the carrier, the weak ligands are replaced by the enzyme molecules [12,15]. The binding can be easily reversed either by the introduction of a ligand with greater affinity for the metal ion (ethylene diamine tetraacetate, EDTA) or by addition of excess of a competing ligand [16].

Advantage: The reversal of the binding provides easy regeneration of the support without any appreciable effect on the yield. The main advantage over other reversible enzyme immobilization methods is that the binding is reasonably strong due to which enzyme-leakage can be maximally restricted. A variety of chelating anions can be provisionally attached to the matrix followed by metastable attachment of the enzyme to the support [15].

Disadvantage: The reagents involved may not be safe for the production of food products and may cause health hazards. Reduction in enzyme activity due to interaction with the metal ions at the active sites of the enzymes is also a major drawback of this process.

Carriers used: The supports used are mostly organic materials, usually cellulose, chitin or silica-based carriers. They are activated as the enzymes bind to their nucleophilic groups by coordinate bonds. Transition metal salts or hydroxides may also serve as carriers [12].

Disulphide bonding

This technology involves the formation of disulphide bonds

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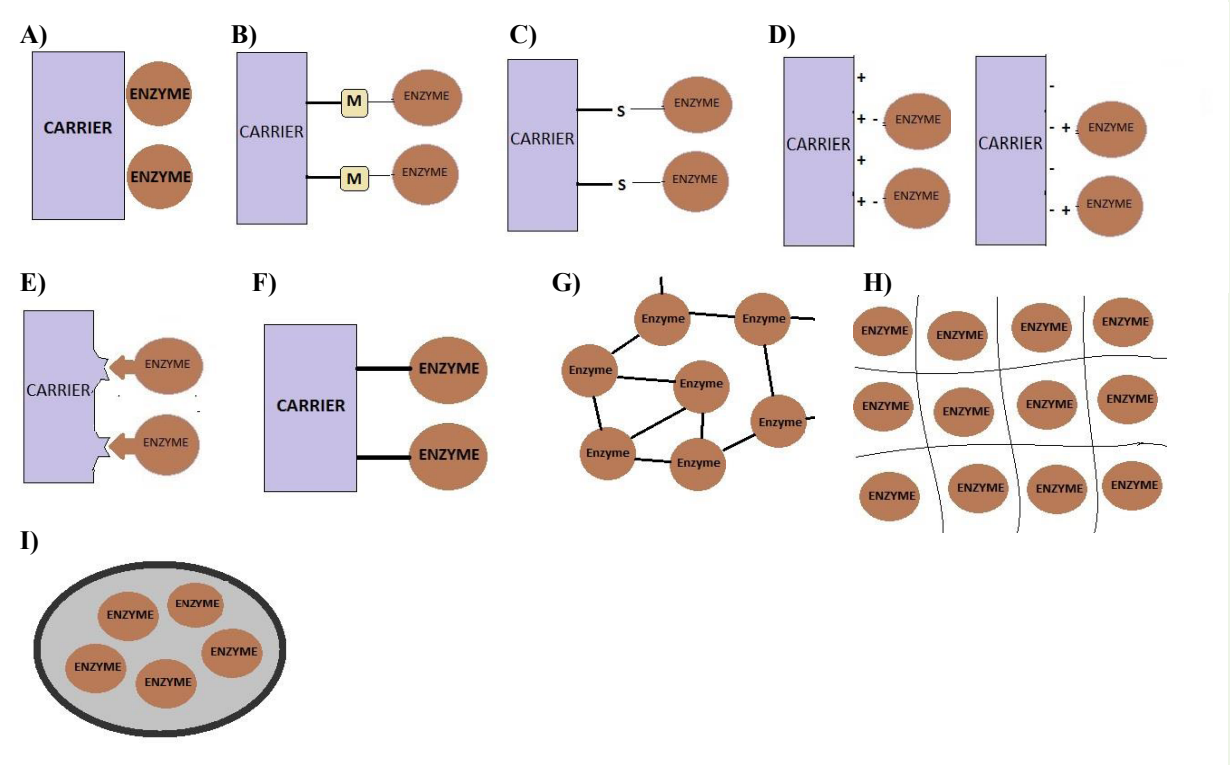

Figure 1: Variations in enzyme immobilization technique; (a) adsorption, (b) chelation, (c) disulfide bonding, (d) ionic binding, (e) affinity binding, (f) covalent binding, (g) crosslinking, (h) entrapment and (i) encapsulation.

between the enzyme and the matrix. Though it is a form of covalent bonding (an irreversible enzyme immobilization method), it is classified as a reversible technique because of the ease of reversal of the binding (Figure 1c).

Principle: The enzyme immobilization step requires formation of disulphide linkage between the carrier and a free thiol group, usually on cysteine residues. The disulphide bond is reasonably stable, especially under physiological conditions (at which many enzymes function). The binding can be reversed by the addition of reagents such as dithiothreitol (DTT) under mild conditions, or by altering the pH [12].

Advantage: Proper maintenance of pH and temperature can restrict enzyme-leakage, as the disulphide bonds are sufficiently stable. The activity of the thiol group can also be altered with pH [12].

Disadvantage: In a reaction mixture, the pH and substrate concentration constantly change as the reaction progresses. These may alter the enzyme binding, consequently leading to enzyme loss.

Carriers used: Supports used are generally inert substances like silica, which are chemically activated by agents such as iodoacetate or maleimide [12,17]. Alternately, the carriers used may also be activated by a method called photonic induction [18].

Ionic binding

This is a simple reversible mode of immobilization of proteins, which involves ionic interaction between the enzyme and the support (Figure 1d).

Principle: The support used is generally charged, such that the protein to be bound has an opposite charge. The enzyme is therefore bound to the support via ionic interactions. It can be easily reversed by altering the pH or 'salting out' of the enzyme [7].

Advantage: It is very easy, inexpensive and requires simple inputs for reversal of the binding. To maintain an optimum pH during the reaction tenure, easy manipulation of the acidity or alkalinity in the reaction mixture can be performed, as the matrix which immobilizes the enzyme is stably charged [19].

Disadvantage: The presence of the charged support causes several problems like enzyme structure distortion and alterations in enzyme kinetics. High charge has the potential to disrupt the enzyme catalysis. As a result, maximum yield is hindered [20].

Affinity binding

This technique is based on the antigen-antibody interaction (Figure 1e).

Principle: This technique is based on high affinity interaction between biomolecules. The carrier matrix is synthesized specifically for a single type of enzyme and contains antibodies against specific epitopes on the antigen (enzyme) [7].

Advantage: The reaction is highly specific and no contaminants

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are present on the carrier. If the antibody on the support is highly specific for the enzyme, the step of enzyme purification can be bypassed. Enzymes from an impure solution can also specifically attach to the matrix. Maximum activity of the enzyme is also ensured if the antibody is targeted at an epitope away from the activity site [21].

Disadvantage: The method involves use of specific antibodies, which are generally very expensive.

Irreversible Enzyme Immobilization

Reversible immobilized enzyme technology cannot fulfil the aim of using an immobilized enzyme on a long term basis. Irreversible immobilization involves strong chemical bonds and particularly serves to maintain reasonable stability of the enzymes over a long period of time. Most industries use enzymes immobilized by these methods, thereby allowing continuous processing of the substrates without the need of replacing the enzyme very often. Different techniques classified as irreversible enzyme immobilization are discussed in the following sections.

Covalent Binding

Covalent bonds are highly stable and hence, covalent binding ensures that the enzyme is strongly bound to the support (Figure 1f). It has been used in a number of industries, since 1973, most prominently in the synthesis of 6-aminopenicillanic acid from Penicillin G, which utilizes penicillin acylase covalently bound to Sephadex G-200 [11].

Principle: It involves the formation of a covalent bond between the support and the side chains of the amino acids of the enzyme, most commonly lysine (ε-amino group), cysteine (thiol group), aspartic acid and glutamic acid (carboxylic group), hydroxyl group, imidazole group, phenol groups, etc. [11]. These groups are nucleophiles and tend to bind to electrophilic groups of the support. A wide variety of reactions have been developed, depending on the functional groups available on the matrix. For coupling of the enzyme to the support, it is often necessary to 'activate the support', i.e., modify the support so as to make it bind to the enzyme more efficiently. The activation methods in general can be divided into two main classes, (i) addition of a reactive group to the support polymer to activate it, and (ii) modification of the polymer backbone to produce an activated group. The activation processes are generally designed to generate electrophilic groups on the support. This allows the support to react with the strong nucleophiles on the proteins, allowing stable immobilization [7].

Advantage: The binding involves covalent interactions and is strong. Hence, leaking of the enzyme into the reaction mixture is totally prevented. This prevents mixing of the enzyme with the product, thereby reducing contamination and the cost of purification [7]. The covalent binding also stabilizes the enzyme in specific protein orientations, and may promote higher specific activity [14].

Disadvantage: The covalent bond formed between the support and enzyme may involve the amino acids of the active site of the enzyme, which may lead to significant loss in activity. Since the method is irreversible, the support cannot be recycled, as the enzymatic activity declines. The support along with the bound enzyme has to be discarded [7].

Carriers used: The supports used are generally stable and easily available and are activated by the appropriate reagents. The common supports used are cyanogen bromide (CNBr)-activated Sephadex or CNBr-activated Sepharose. Other common carriers include activated forms of dextran, cellulose, agarose, etc. [11]. Artificial matrices include Polyvinyl chloride, ion exchange resins and porous glass [12].

Crosslinking

It is an irreversible method of enzyme immobilization (Figure 1g). It is different from other techniques in the sense that it does not require a support for the immobilization. There are two methods of cross linking in use, (i) Cross Linking Enzyme Aggregate (CLEA), and (ii) Cross Linking Enzyme Crystals (CLEC). Both CLEA and CLEC are modifications of a primitive method, where cross linking agents such as glutaraldehyde (which react with the amino group on the protein) were used. The CLEC or CLEA are added to the reaction mixture and can be later removed from the mixture during product purification. Hence, unlike the other systems of enzyme immobilization, the immobilized enzyme is not bound to any matrix, but is present in the reaction mixture, albeit in an immobilized form. The two methods are described below.

A. Cross Linking Enzyme Crystals (CLEC)

Principle: In this method, glutaraldehyde is used to crystallize the enzyme. Hence, enzyme crystals are obtained with the help of the cross-linking agent. The CLEC, upon addition to the reaction mixture, catalyzes the reaction with reasonably high efficiency [12].

Advantage: The CLEC are very stable and are not easily denatured by heat or organic solvents. They are moderately resistant to proteolysis. They have a manageable size and stability in operating the bioreactor and can be recycled [12, 22]. They also give enhanced enantioselectivity to particular forms of their substrate [23].

Disadvantage: Highly purified enzyme produced through a standard protocol of crystallization is required for the preparation of CLEC. These requirements involve a lot of time and expenses. Diffusion of substrate and product is limited with increase in size of the aggregate [24].

B. Cross Linking Enzyme Aggregates (CLEA)

Principle: CLEA is an improved version of CLEC production and aims at nullifying the disadvantages of CLEC. While CLEC requires the formation of crystals, CLEA could work in aqueous solutions. Addition of salts, organic solvents or non-ionic polymers results in the formation of enzyme aggregates which retain their catalytic properties. These aggregates are called Cross Linked Enzyme Aggregates (CLEA) [12] (Figure 2).

Advantage: It is cheaper, easier to perform and has a wide range of applications. For multi-enzyme catalysis, it is possible to synthesize CLEA having more than one enzyme in the aggregate (called combi-CLEA) [23].

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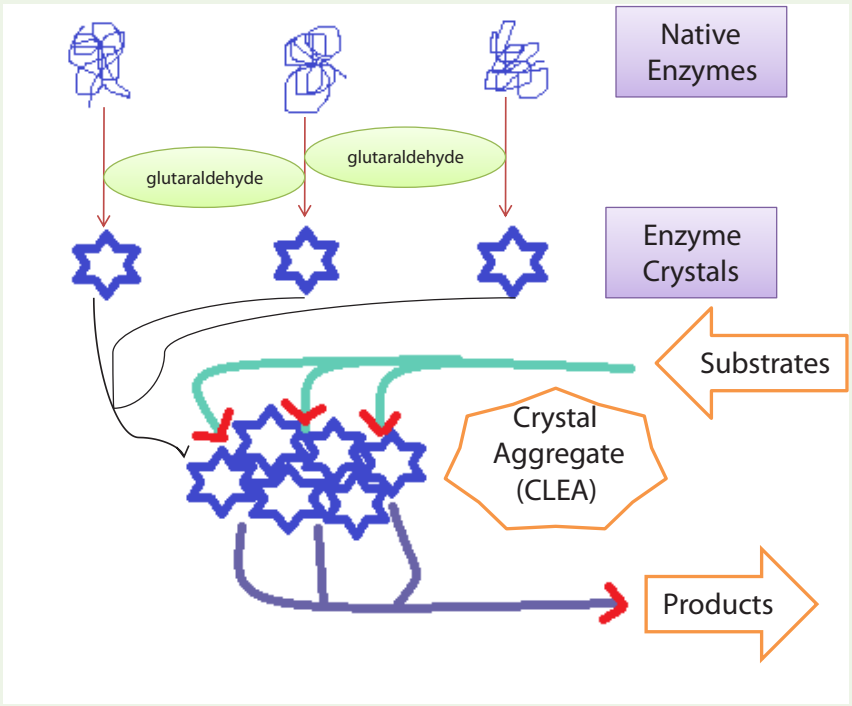

Figure 2: The mechanism of action of CLEA via formation of enzyme crystal aggregates.

Disadvantage: The size of the derived aggregates is small and often similar to the size of the substrate or product; this may cause severe inconvenience during product purification [25]. Diffusion of substrate and product is limited with increase in size of the aggregate [24]. The CLEAs are often found to be fragile and tend to exhibit low stability in stirred tank fermenters or packed bed reactors [23].

Crosslinking agents used: For most enzymes, the crosslinking agent used is glutaraldehyde, which is cheap, stable and easily available. However, glutaraldehyde partially or totally inactivates some enzymes. For such biomolecules, alternative cross-linking agents such as dextran polysaccharide, bis-isocyanate, bis-diazobenzidine, diazonium salts and functionally inert proteins, such as bovine serum albumin (BSA) should be preferred [12,22].

Entrapment

Entrapment is another prominent technique of irreversible enzyme immobilization, where the enzyme is immobilized by entrapping it within a support matrix or within fibres (Figure 1h).

Principle: Enzymes, being large macromolecules, tend to be larger than the substrates or products. Thus, the enzyme is immobilized within a matrix of appropriate pore size to allow only the substrates and products of a diameter smaller than the matrix pore size to diffuse in and out of the mesh respectively [13]. The enzyme size-to-pore size of support is a deciding factor in selecting the support. Smaller the pores, lesser the enzyme entrapped, while larger the pores, more the leaking of the enzyme. Hence, accurate pore size selection of the support is crucial [12].

Advantage: The method is fast, cheap and easily carried out under mild or physiological conditions. As the enzyme remains confined within a matrix, it is protected from contamination by microbes, proteases or other enzymes [13].

Disadvantage: The meshwork of the matrix cannot support a huge volume of enzyme molecules and can lead to enzyme inactivation. Hence, the process can be costly at times. The rate of diffusion of the substrate and product dictate the reaction rate. This is because unless the substrate molecules diffuse into the mesh, the reaction will not be initiated and according to Le Chatelier's principle, the reaction rate does not reach a peak unless the products sieve out [12,13].

Matrix used: Common polymers used for enzyme entrapment include alginate, carrageenan, collagen, polyacrylamide, gelatin, silicon rubber and polyurethane [26].

Encapsulation

Encapsulation can be regarded as a special type of entrapment where the enzyme is immobilized by entrapping it in a spherical semi-permeable membrane (Figure 1i).

Principle: The basic underlying principle is the larger size of the enzyme as compared to substrates or products. When the enzyme can be entrapped or occluded within a semi-permeable membrane, it would allow the small substrate molecules to diffuse in and the product molecules to diffuse out. However, the enzymes being much larger, cannot diffuse through the membrane and remains within it. Hence, the enzyme is restricted within the membrane, although it is free floating inside the capsule [12]. The permeability of the membrane is controlled according to the enzyme being immobilized. Encapsulation is achieved by one of the two methods, (i) Coacervation

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(allowing the polymer to separate out enzyme microdroplets in a water immiscible solvent), and (ii) Interfacial polymerization (when a hydrophobic monomer is added to an aqueous solution of enzyme and another monomer which has been dispersed in a water immiscible solvent. This promotes polymerization at the interface of the two droplets and hence around the enzyme).

Advantage: Encapsulation within a membrane maintains the enzyme structure in its native form and prevents leakage of the enzyme, protecting it from the harsh conditions of the medium. Multi-enzyme encapsulations can also be created by trapping more than one enzyme within a membrane [27].

Disadvantage: As mentioned earlier with entrapment method, the diffusion of substrate and product across the membrane controls the reaction rate. The pore size needs to be maintained accurately to prohibit enzyme leakage (if the pore is too large) or poor loading of the enzyme (if pores are very minute). This technique is non-recommendable for reactions involving substrate and enzyme molecules of similar diameters as an optimum pore size cannot be selected in such cases [12].

Biopolymers used: A variety of biopolymers like alginate, maltodextrin, cellulose, chitosan, etc. are available which can be used for the purpose of encapsulation of an enzyme. These polymers form a single layered capsule around the enzyme. Double-layer microcapsules, built of two distinct types of polymers, are also popular. The most common ones are composed of chitosan, Poly-L-lysine, Polyvinyl acetate, gelatin and boric acid [6].

Whole cell immobilization as an alternative strategy to enzyme immobilization

Cells may be described as living biocatalysts. Every cell contains different enzymes, which can be used to catalyze a large variety of reactions. Some of these reactions yield products that are required in their life processes, such as energy production and anabolism. When reactions are catalyzed by cells in a bioreactor, the cells are generally not immobilized, and remain floating in the medium. Hence, they often have to be filtered out and discarded during purification of products. In nature, microorganisms are often found growing on a substrate. Biofilms are surface-attached microbial communities consisting of multiple layers of cells embedded in hydrated matrices. This allows bacteria to grow attached to the substrate, and especially in niches where the surrounding medium is not static or stable, e.g., on rocks under flowing rivers, on the walls of pipes, teeth, etc. [28]. It is often seen that bacteria bound to a surface grow and proliferate better. Hence, this principle was put to use in immobilizing cells, and then use them for human activity. In this technology, the cells remain bound to a surface, while the nutrients (essential for cells to survive), substrates and products diffuse in and out of the cells (owing to the semi-permeable nature of the cell membrane) [28]. Whole cells may be immobilized either in a viable or non-viable form [29].

Principle: The discussion on immobilized cells to be used as biocatalysts constitute two major points as mentioned below.

(i) The cells need to be properly attached to a suitable support. Different techniques can be adapted for the same and a few techniques include immobilizing the cells under a porous support like polymeric gels, attaching them to a micro carrier surface or entrapping them behind membranes [28]. The last approach is less favoured, as it creates a constraint of space, and the rate of the reaction is limited by the diffusion parameters (the nutrients, substrates and products have to diffuse through two membranes - the external membrane as well as the membrane of the cells). The strategy used commonly is attaching them to a carrier; several motifs on the cell membrane or wall of the cell being immobilized are utilized. Targeting the characteristic epitopes on the membrane of commercially useful microorganisms can allow efficient binding of the cells to the carrier [29].

(ii) Easy diffusion of the substrates and products within the cells is essential for economical production at the industrial scale. The cells may be permeabilized to act as better intracellular source of enzymes and this may be achieved by physico-chemical techniques. Physical techniques include freezing and thawing. Chemical techniques utilize organic solvents (toluene, chloroform, ethanol, butanol) and mild detergents (N-Cetyl-N, N, N-trimethyl ammonium bromide, i.e., CTAB and digitonin) to increase the membrane porosity [29]. However, to avoid unwanted cell lysis, low dosage of the chemicals is advocated. Permeabilization is only necessary for enzymes that work in the cytoplasm. For enzymes active in the periplasmic space such as catalase (in yeasts), urease, Penicillin G acylase (in bacteria), the cells can be utilized without permeabilization. Hence, novel techniques of using intracellular enzymes include their transport into the periplasmic space, via genetic engineering. Anchoring proteins attach the enzymes, either to the exterior of the cell membrane or the interior of the cell wall. The anchoring proteins with a characteristic LPXTG domain aid enzymes to remain in the periplasmic space via attachment with the cell wall [29]. The recombinant *Escherichia coli* expressing *organophosphorous hydrolase* (*OPH*) gene has been created in this way [30].

Advantage: The conditions of the cytosol or resident organelles are the optimum for respective enzymes. Viable cells continue to divide and naturally replenish the enzymes inside a bioreactor. Continuous fermentation processes tend to suffer from the problem of reduction in cell density due to washing away of cells. This can be overcome by using immobilized cells for the process. The cell density of the culture can be pre-determined, as the number of cells to be immobilized can be controlled [29]. The major advantage is that this process is more economical than enzyme immobilization, because it is far more expensive to get a purified enzyme as compared to a cell culture.

Disadvantage: Cell immobilization is far less expensive as compared to enzyme immobilization. Hence, it may seem reasonable to utilize cell immobilization over enzyme immobilization. However, this method involves certain disadvantages which have been discussed as follows:

(i) The rate of catalysis is highly limited by the rates of diffusion of substrate and product across the membrane, as the enzymes remain entrapped within the cells.

(ii) For aerobic cells, proper oxygenation is vital for survival and growth. The dissolved oxygen needs to be maintained at a high level,

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as the cells lie embedded on a solid surface (usually inside the media).

(iii) The matrices may tear due to excessive proliferation of the cells [31].

(iv) Immobilization can only be used for cells that release out the products to the exterior. Since the cells are immobilized, it is not possible to extract the cells and lyse them for releasing the products.

(v) A cell is a reservoir of a myriad of enzymes. So, there are chances of cross-reaction of the substrate with unwanted enzymes, or the product can be converted to some unwanted forms.

Enzyme Modification vs. Enzyme Immobilization

Genetic modulations and experiments have created variations of enzymes which better catalyse reactions than their normal counterparts. Such modifications, though seem costly during initial implementations, are actually cost-effective in the long run, as they produce higher yields than their normal counterparts. Enzyme modification is to be used primarily when the native form of the enzyme has either very low activity and is extremely labile or readily degraded by the conditions in which the reaction is to be carried out. The enzyme immobilization tries to prevent its mobility, while still retaining its catalytic activity, while enzyme modification focuses on modifying its activity, sensitivity to various chemicals and altering the specificity. This technique seems to maximise the industrial efficiency of an enzyme and tries to retain its catalytic efficiency to the maximum. It fixes usable enzymes to a support, so that they are not lost during purification [6].

Applications of immobilized enzymes

1. Immobilized enzymes as biosensors

A biosensor is an analytical device that responds to an analyte in an appropriate sample and interpretates its concentration as an electrical signal, via a suitable combination of a biological recognition system and an electrochemical transducer [32]. It contains an optical, electrical, chemical or mechanical as well as a biological component, usually an enzyme, antibody or nucleotide. Biosensors should be able to detect even traces of biological activity and must be able to distinguish between two different living entities as well. The idea of using immobilized enzymes is highly sensible, as enzymes show high sensitivity, rapid detection, reasonable costs, etc. As already discussed, enzyme immobilization also prevents enzyme-loss, maintains the stability of the enzyme structure, enhances shelf life and provides solid disposable detection kits that facilitates immediate and on-field detection. These techniques are applicable in the preparation of medicines. Their high specificity might be used in detection of very minute quantities of pathogens and toxins in food, water or even within human body [4]. The most commonly used example of an immobilized enzyme, being used as a biosensor is that of the glucose oxidase-based electrodes, that are used for monitoring the level of glucose in a system [5]. The Enzyme Linked Immunosorbent Assay (ELISA) is another popular diagnostic tool, used as a biosensor for especially detecting viral infections. Another popular kind of biosensor being put to use nowadays involves the principle of enzyme inhibition. It uses an analyte such as organophosphorous pesticides and derivatives of insecticides or heavy metals. These toxic analytes are chosen on the basis that they would inhibit normal function of enzymes and that the percentage of inhibited enzyme would be proportional to the quantity of the toxic analyte. Hence, it gives an estimate of toxin quantity [4]. Biosensors based on immobilized enzymes are even used in astrobiology, for detecting the presence or absence of life in other planets. Proteins characteristic of existence of life may be detected by the biosensors. This is based on drilling the ground to a depth of about 1.5-2 m to search for an amino acid signature of life that got extinct about 3 billion years ago on Mars. A microfabricated capillary electrophoresis device, called Life Marker Chip (LMC), that utilizes immobilized enzyme for the determination of amino acid chirality was developed and incorporated into the UREY instrument selected as part of the Pasteur payload for the ESA (European Space Agency) ExoMars mission [28].

2. Production of antibiotics

Classical methods of antibiotic production do not involve cell cultures. Attempts are being taken to overproduce such secondary metabolites by keeping the cells under stress. As the immobilized cells are confined to a small localized region, it is easier to induce stressful conditions required for the production of secondary metabolites. Immobilized β-acylase shows better efficiency in the hydrolysis of Penicillin G. Enzymatic production of Cephalexin using immobilized Penicillin G Acylase also shows promise [4].

3. Biodiesel production

Ethanol and biodiesel have shown the ability to replace petroleum and fossil fuels. On the basis of extent of energy retained, these hydrocarbons play pivotal roles as biofuel. The alternative hydrocarbons are required, as the traditional fuel is gradually depleting. Biologically derived fuels are eco-friendly, causing lesser environmental hazards and are economically feasible [33]. Biodiesel can be produced from vegetable oils, animal oils, microalgal oils and waste products of vegetable oil refinery through trans-esterification of triglycerides and do not emit oxides of sulphur, halogens or other toxic gases upon burning [34]. Hence, emphasis on the synthesis of biodiesel is very high. Classical methods use chemicals such as H_2SO_4 or NaOH and require a large input of energy and water. Use of immobilized lipase for the production of biodiesel is gradually gaining importance. The method is easy, economic and environment-friendly, allowing easy recovery of glycerol, shows excellent catalytic activity and is stable in non-aqueous media [5].

4. Bioremediation

Industries release harmful chemicals as effluents into water bodies or into the soil, leading to widespread water pollution. Apart from toxic heavy metals like arsenic and mercury, industrial effluents often contain aromatic azo-dyes. Upon anaerobic transformation, these produce colourless aromatic amines that are carcinogenic and pose a great threat to human health. Use of immobilized enzymes for the purpose provides some promise. Peroxidases from bitter gourd immobilized on a support have been effective in decolorizing reactive textile dyes. Immobilized laccases and polyphenyl oxidases prove effective in detoxifying phenolic compounds in water bodies [4]. The

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immobilized laccase enzyme has the ability to degrade anthracinoid dye, Lancet blue and Ponceau red [13]. The Single Enzyme Nanoparticle (SEN) is particularly used to chelate heavy metals from polluted areas. In SEN, the enzyme is coated by a nanometre thick material. The SEN retains its activity even in extremely harsh conditions [13]. High Efficiency Nano-Catalyst Immobilization Technology (HENCI) is a promising new technology based on nano-scaled catalysts, which can be used to degrade rare yet dangerous pollutants. Owing to their size and efficiency, they might be very useful in removing such pollutants to a very minute level. However, they are reasonably expensive and efficient supports to immobilize them in large quantities are yet to be found [28]. Soils contaminated with pesticides may be treated with immobilized enzymes capable of degrading many organophosphorous compounds [28].

Therapy

Immobilized enzymes find immense application in the fields of therapeutics and pharmaceutical industry.

Treatment of kidney disorders

Kidney disorders often have several painful consequences, including gout. Gout is characterized by inflammation of joints due to the accumulation of uric acid. It may be because of decreased secretion of urate by the tubules due to excess anions, or due to increased tubular reabsorption. Present day treatment of gout involves treatment with an anti-inflammatory agent, followed by uricosuric agents to enhance renal excretion of urate. A novel method involves the use of uricase immobilized to mesoporous silica. The enzyme-entrapped mesoporous silica can be used as a transdermal patch. The uricase converts uric acid to allantoin, which is about 100 times more water soluble than urate [35].

Treatment of liver disorders

Hepatic failure is very dangerous, and if untreated may result in death. Liver performs a huge array of vital metabolic functions in our system, including detoxification of several toxins. When the liver fails to perform its tasks, the toxins accumulate and may lead to hepatic coma. The enzymes responsible for hepatic detoxification, e.g., alcohol dehydrogenase have been purified from rabbit liver, immobilized to a hemocompatible form of agarose matrix and tested to show fair stability and activity for a prolonged period of time. This could be used for treatment of hepatic failure or other liver disorders [36].

Treatment of thromboembolic disorders

Thromboembolic disorders refer to the fatal syndrome in adults where blood clot formed at one location in a blood vessel is carried by the blood to another location, where it blocks a blood vessel. Immobilized streptokinase may be used for the treatment of such disorders. They provide prolonged fibrinolytic activity and hence can be a valuable asset in the treatment of anticipated thromboembolic disorders [28].

Treatment of inflammatory manifestations

Superoxide dismutase (SOD) and catalase (CAT) have been immobilized in biodegradable microspheres to obtain efficient delivery systems. The enzymes were found to remain stable throughout in the immobilized state. SOD was also seen to be released slowly and uniformly over a prolonged period of time, around two months in *in vitro* assays. Hence, these could be used for the treatment of inflammatory manifestations, as in rheumatoid arthritis and various intra-articular joint diseases [28].

Treatment of inborn errors of metabolism and cancer therapy

Inherent errors of metabolism are caused when an organism lacks the ability to produce or utilize a particular substrate or component. This is mostly caused due to defective enzyme(s) in the metabolic pathway. This can be treated by encapsulating the enzyme (for which the patient is deficient) with erythrocytes (of the patient or someone with the same blood group). The erythrocytes would then serve to carry the enzyme throughout the system without generating an immune response. Cancer therapy and the treatment for various other diseases, using nanoparticles as carriers are also being researched upon. These could work at delivering the drugs to their exact targets with great accuracy [13].

Detergent synthesis

Synthetic detergents can be produced using immobilized enzymes for better removal of dirt and stain. Enzymes such as proteases, amylases, cellulases, etc. are immobilized using the granulations of the detergent. These enzymes are relatively stable under harsh conditions of washing and show good activity [5]. Lipases are used to remove stains caused by fats and oils. Proteases remove stains caused by proteins, such as those caused by blood, egg and sweat. Amylases remove stains caused by carbohydrates such as chocolate and gravies. Cellulase is used to improve the softness and colour brightness of cotton clothes [13].

Food Industry

Immobilized enzymes play a key role in the food industry and have a wide range of applications. We have briefly discussed the applications in the following sections:

Brewing industry

Use of immobilized yeast cells allows the increase in the cell concentration in a definite space of the fermenter. It allows retaining the cells and simplifies the purification step.

Dairy industry

Immobilized enzymes are often put to use in the dairy industry to obtain the starting components and allow various reactions, such as cheese and yoghurt production and prevention of growth of psychotrophic bacteria in milk. Immobilization can also alter both the lactose and citrate metabolism and promote further nutritional enrichment of dairy products [5]. Immobilized lipases have allowed the incorporation of linolenic acid (an anti-carcinogen) into dairy products [37].

Meat industry

Using immobilized cells could aid in meat fermentation and

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retain a continuous cell line. It creates a starter culture that is cheaper and provides a continuous cell line for the process [5].

Alcohol industry

Immobilized cells are used for the production of alcohol. Alcohol-producing bacterial cells are immobilized and the fermentation is carried out in a bioreactor. It is observed that immobilized cells exhibit a higher rate of product formation as compared to the cells that are not immobilized [5].

Organic acid production

Organic acids such as citric acid, malic acid, propionic acid, butyric acid, gibberellic acid and succinic acid may be synthesized using immobilized cells. Citric acid is generally synthesized through *Aspergillus niger*. Increased fungal growth disrupts uniform oxygen distribution and is antagonistic to high product yields. Immobilized fungal cells show reduced proliferation and hence lower utilization of oxygen, thereby prolonging organic acid production [5].

Syrup production

Enzymes like β-galactosidase are immobilized to a substrate and are used generally for lactose fermentation [13]. The first large industrial process to utilise immobilized enzyme was the production of high fructose corn syrup by Clinton Corn Products and used glucose isomerase adsorbed to DEAE-Cellulose as discussed earlier [11].

Textile industry

Common immobilized enzymes such as cellulose, amylase and pectinase derived from microbes have a variety of roles in textile industry. Immobilization makes the enzyme withstand the harsh conditions of the cloth processing. The processes such as scouring, treatment of wools, bio-polishing and denim finishing are achieved using immobilized enzymes [13].

Enzyme Immobilization and Nanotechnology

With the development of nanostructures such as nanofibres, carbon nanotubes and gold nanoparticles, Nanotechnology seems to be offering better and more efficient support. They provide a large surface area for binding of the enzyme, resulting in a net increase in both enzyme loading and enzymatic activity per unit volume. The enzymes are taken up by a process called nano-entrapment, which leads to discrete nanoparticles. Owing to their porosity and high surface area, nanostructures could more often be considered for usage as carriers. While enzymes bound to the nanostructures by adsorption show a little leaking of the enzyme, those bound by covalent linkage exhibit almost zero leaking [5].

Further technology developments

Efficient simultaneous production of D-hydantoinase (Dhase) and D-decarbamoylase (Dcase) has been recently reported by whole cell immobilization of the recombinant *E. coli* strain LY13-05. Fermentation for 12 h with 30 g L^{-1} DL-p-hydroxyphenyl hydantoin (DL-HPH) as the substrate for the immobilized strain caused the broth cell density to reach 1.9 g L^{-1}. The yield of D-HPG was 29 g L^{-1}, that of D-p-hydroxyphenylglycine (D-HPG)/HPH was 97% and the specific productivity was 1.3 g $(g.h)^{-1}$ with the productivity as 2.4 g $(L.h)^{-1}$. The advantages observed in this process was the high substrate conversion efficiency during the production of D-HPG and the specific yield rate, which were better than the yield of D-HPG from other reported strains. Conversion efficiency and yield was also reported to be higher for the recombinant strain in comparison to that of the free cells. Moreover, the immobilized strains had better thermal stability, higher repetition application time and longer storage duration, which will supposedly have wider implications for industrial productions [38].

Immobilized enzymes have always posed as a major cost factor in the utilization of heterogeneous catalysts on an industrial scale. The formation of foreign peptides associated with the oil body in plant seeds has been strategized. Such production can be manipulated by genetically modifying rapeseeds at relatively low cost. Floatation centrifugation can be used to easily separate out the oil bodies. The catalytic activity of the reporter β-glucuronidase expressed on the oil body surface is comparable to those of free β-glucuronidase enzyme [39]. The United States Environmental Protection Agency has been engineering stable immobilized enzymes for the hydrolysis and transesterification of triglycerides. The main improvements undertaken in this project were (i) catalyst improvement by increasing the specific surface area of the immobilized matrix within a range of macropore size; (ii) characterization of the used materials in order to provide feedback to the catalyst improvement studies regarding pore size distributions, specific surface areas and enzyme distribution within the alcogel; (iii) studies related to the bioreactor to improve reactivity, reusability and stability of the used immobilized enzymes (http://cfpub.epa.gov/ncer_ abstracts/index.cfm/fuseaction/ display. highlight/abstract/6727).

Multi-Enzyme Immobilization

Cells are complex machineries and require many enzymes to perform the life functions. Multiple enzymes work in a cascade to perform a set of complex reactions within all living cells. However, most attempts to utilize more than one enzyme in a bioreactor often lead to failure due to the sensitivity of the enzymes as well as cross reactions. Enzyme immobilization promises to tackle the problem and deliver multi-enzyme immobilized systems, mostly by the technique of encapsulation [8]. The enzymes may retain their activity and have their active sites close to each other. This can allow a mechanism similar to substrate channelling without the diffusion of the intermediates (Figure 3). However, multi-enzyme immobilized systems face several challenges. The enzymes should have enough spatial separation to prevent steric hindrance between the enzyme subunits that would distort their structure, but should be close enough to ensure maximal activity. The support should be such that it allows maximal loading of the enzymes, as well as ensures maximum enzyme activity. A biosynthetic approach co-localizing the two enzymes on nanocarriers to mimic multi-enzyme complex systems that occur in nature can potentially improve the product yield significantly. Different polymeric nanocarriers are being investigated to act as supports for multi-enzyme systems [40].

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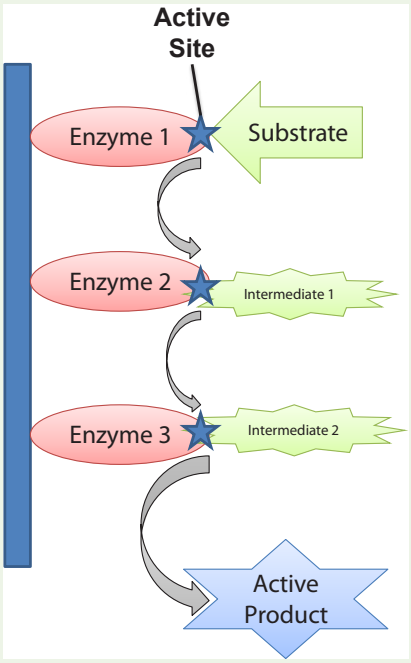

Figure 3: Schematic representation of the operation of a multi-enzyme immobilization system; product formation occurs through shifting of the intermediates from one enzyme active site to another, analogous to substrate channelling.

Conclusion

The specificity of the enzymes as well as their ability to catalyze reactions in mild conditions makes them very precious. Immobilized enzymes often show remarkable stability, high levels of activity and greatly reduce the cost of product processing [8]. However, immobilization does cause a slight loss of activity in most cases. The costs of enzyme purification and immobilization are often high and result in increasing the cost of the overall reaction [7, 24]. These disadvantages need to be addressed to improve the overall reaction and make it more feasible. There is plenty of scope for further research to develop better methods of immobilization as well as better carriers. Nanotechnology is one of the more promising fields that attempts to provide an efficient substrate. Identification of effective immobilization techniques on appropriate supports could answer several pressing questions that challenge industrial development and human welfare, viz., bringing down the costs of food products, coming up with more efficient disease detection kits, strategies to clean up contaminated water bodies and even finding a way to cure/control cancer. The possibilities, opportunities and prospects in the field of enzyme immobilization are therefore endless.

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