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Research Article

A Review on Methods, Application and Properties of Immobilized Enzyme

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Abstract

Immobilized enzymes are widely used for variety of applications. Based on the type of application, the method of immobilization and support material can be selected. The immobilized enzymes can be separated from the reaction mixture and reused and also immobilized in order to prevent the enzyme from being exposed to harsh conditions, high temperature, surfactants, and oxidizing agents etc. the immobilized enzymes are also widely used in food industry, pharmaceutical industry, bioremediation, detergent industry, textile industry, etc. Enzyme immobilization improves the operational stability and is also due to the increased enzyme loading which causes the controlled diffusion. Several hundreds of enzymes are immobilized and used for various large scale industries. Immobilization technique reduces the effluent treatment costs and this paper reviews the methods and applications of immobilized enzymes.

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Introduction

An enzyme derived from an organism or cell culture that catalyses metabolic reaction in living organisms and /or substrate conversions in various chemical reactions. The enzymes are the potential catalyst works in mild temperature, pressure, pH, substrate specificity under suitable reaction conditions and for the production of desired products without any intermediate products as contaminations for these advantages enzyme are used in variety of application such as cosmetics, paper industry, textile industry, food industry, pharmaceutical industry, laundry and in detergents etc [1-5]. The biotechnological method of producing enzyme is expensive; hence new methods have been implemented to reduce the cost. The enzymes have various other limitations such as low stability, highly sensitive to the process conditions and these problems can be overcome by the immobilization techniques [6-8]. Immobilized enzymes are being used since 1916, when Nelson and Griffin discovered that invertase when absorbed to charcoal has the ability to hydrolyse the sucrose [9]. The possibility of immobilized enzyme for its reuse and stability was identified by Grubhofer and Schelth, reported the covalent immobilization of several enzymes [10]. The repeated assay can be done with the immobilized enzyme which reduces the cost of assay and the reuse of enzyme process is also very simple and it can be attained through ultrafiltration technique. In earlier days the immobilization of enzymes are carried out using biotechnological method , but now due to rapid growth of nanotechnology, the synergistic interaction of two technologies, the development of immobilized enzymes on various nanomaterials using conventional methods such as covalent and adsorption attachment [36] **(Table 1)**. B M Brena and F Batista have classified enzyme immobilization as Irreversible enzyme immobilization and Reversible enzyme immobilization methods. Irreversible enzyme immobilization includes covalent binding and Entrapment. Reversible enzyme immobilization includes adsorption, ionic binding, affinity binding and metal binding [2]. Immobilization of enzyme by enzymatic process is recently identified by researchers in order to avoid harsh immobilization.

Enzyme Immobilization Methods

Covalent Binding:

Covalent binding is a conventional method for immobilization; it can be achieved by direct attachment with the enzyme and the material through the covalent linkage [37]. The covalent linkage is strong and stable and the support material of enzymes includes polyacrylamide, porous glass, agarose and porous silica [38]. Covalent method of immobilization is mainly used when a reaction process does not require enzyme in the product, this is the criteria to choose covalent

immobilization method. This covalent binding of the enzyme with the support material involves two main steps such as, the activation of the support material by the addition of the reactive compound and the second one is the modification of the polymer backbone to activate the matrix **(Figure 1).**

Table 1 Developmental Phases of Immobilized Enzymes

Phase	Years	Development
Early Phase	1916-1940	Glass [11], Alumina[12], Hydrophobic compound coated glass[13].
Underdeveloped Phase	1950	Non-specific physical adsorption of enzymes on solid carriers e.g amylase on activated carbon, bentonite or clay [14], chymotrypsin on kaolinite [15] AMP deaminase on silica [16],ribonuclease on the anionic exchanger Dowex-2 [17]
Developing Phase	1960	Entrapment of whole cells in synthetic gel [18], Encapsulation in artificial cell [19], Adsorption-cross-linking [20], Active site titration [21], Cross-linked enzyme (CLE) [22], cross-linked enzyme crystals (CLEC), Immobilization or post-treatment by denaturant [23]
Developed Phase	1970	Many new method subgroups, for example affinity binding and coordination binding and many novel variations have been developed [24]. Increased enzyme loading in order to enhance the activity [25,26]
Post Developed Phase	1980	Encagement (double encagement) [27,28], Covalent multilayer immobilized enzymes [29], Organosoluble lipid-coated enzyme [30], Introduction of genetically engineered tags [31]
Rational Design Phase	1990-till now	Stability and activity in organic solvents [32,33], High enzyme loading and less diffusion limitation [34], Development of single enzyme nanoparticle [35]

The activation step produces the electrophilic group on the support material, so that the support material couples /reacts with the strong nucleophiles on the proteins [2]. For example glutaraldehyde is the activation method, in this reaction the amine group reacts with the activated matrix [39]. The covalent binding is normally formed between the functional group in the support matrix and the enzyme surface that contains the amino acid residues. The amino acid residues involved in the covalent binding are the sulfhydryl group of cysteine, hydroxyl group of serine and threonine [40, 41]. The attachment between the enzyme and the support material can be achieved either through direct linkage or through the spacer arm. The potentiality of using the spacer arm is that it provides the greater degree of the mobility to the enzymes hence the enzymes show the higher activity when compared to the direct attachment.

Entrapment:

Enzymes are occluded in the synthetic or natural polymeric networks, it is a permeable membrane which allows the substrates and the products to pass, but it retains the enzyme inside the network, the entrapment can be achieved by the gel, fibre entrapping and microencapsulation [42]. The advantage of entrapment of enzyme immobilization is fast, cheap and mild conditions required for reaction process. The disadvantage is that limitation in mass transfer. The support matrix protects the enzymes from microbial contamination, proteins and enzymes in the micro environment [43]. Microencapsulation method is that the enzyme molecules are capsulated within spherical semipermeable membranes with a selective controlled permeability [44]. This method provides the large surface area between polymeric material and the enzyme. The drawback of this method is inactivation of enzyme during encapsulation [42].

Adsorption:

This is a simple method of preparing an immobilized enzymes and the materials used for adsorption are activated charcoal, Alumina, Ion exchange resins, this method is cheap and easy for use and the disadvantage is a weak binding force between the carrier and the enzyme [45]. This method comes under carrier bound immobilization and the process of immobilization is reversible. Adsorption is the easiest and oldest immobilization techniques [46]. The interaction between the enzyme and the surface of the matrix through weak forces by salt linkage, hydrogen bonds, hydrophobic bonds, ionic bonds and van der waals forces. Based on the charges of the matrix and the protein arrangements the strongly bound, but not distorted enzyme will be formed [47]. The advantage of enzyme adsorption is minimum activation step and as a result of minimum activation, no reagents required. It is cheap and easy way of immobilization.

Ionic Binding:

The bonding involved between the enzyme and the support material is salt linkages. The nature of this non covalent immobilization the process will be reversed by changing the temperature polarity and ionic strength conditions. This principle is similar to protein-ligand interactions principles used in chromatography [48].

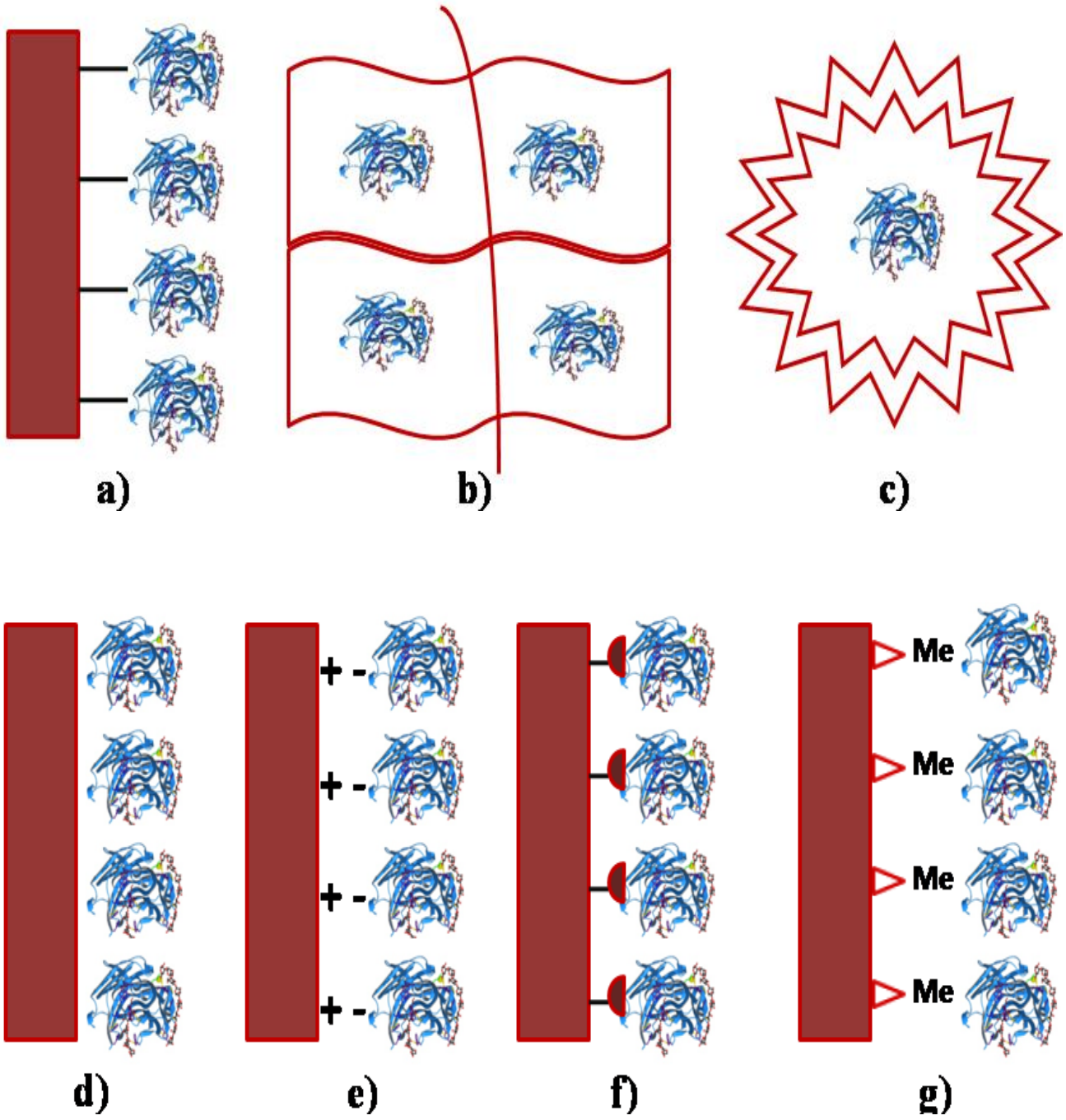

Figure 1 Shows pictorial representation of different Enzyme Immobilization methods a) Covalent Binding b) Entrapment c) Encapsulation d) Adsorption e) Ionic Binding f) Affinity Binding and g) Metal ion Immobilization

Affinity Binding:

The immobilization of enzyme linked to the matrix through the specific interactions. The Two methods are being followed in affinity immobilization. The first method is the activation of the support material which contains the coupled affinity ligand, so that the enzyme will be added. The advantage of this method is the enzyme is not exposed to any harsh chemicals conditions. The second method, the enzyme modified to another molecule which has the ability to bind towards a matrix [49].

Metal Linked immobilization:

In metal linked enzyme immobilization, the metal salts are precipitated over the surface of the carriers and it has the potential to bind with the nucleophilic groups on the matrix. The precipitation of the ion on the carrier can be achieved by heating. This method is simple and the activity of the immobilized enzymes is relatively high (30-80%). The carrier and the enzyme can be separated by decreasing the pH, hence it is a reversible process [64].The matrix and the enzyme can be regenerated, by the process.

Application of the Immobilized Enzymes:

Biomedical Application:

Immobilized enzymes are used in medicine from 1990 [50,51], immobilized enzymes are used for diagnosis and treatment of diseases in the medical field. The inborn metabolic deficiency can be overcome by replacing the encapsulated enzymes (i.e, enzymes encapsulated by erythrocytes) instead of waste metabolites, the RBC acts as a carrier for the exogenous enzyme drugs and the enzymes are biocompatable in nature, hence there is no immune response [52]. The enzyme encapsulation through the electroporation is a easiest way of immobilization in the biomedical field and it is a reversible process for which enzyme can be regenerated [53]. The enzymes when combined with the biomaterials it provides biological and functional systems. The biomaterials are used in tissue engineering application for repair of the defect. The advantage of the enzyme immobilization in biomedical is that the free enzymes are consumed by the cells and not active for prolonged use, hence the immobilized enzymes remains stable, to stimulate the growth and to repair the defect. The cancer therapy is delivery of enzymes to the oncogenic sites have been improved with new methods. The nanoparticles and nanospheres are often used as enzyme carriers for the delivery of therapeutic agents.

Food industry Application:

In food industry, the purified enzymes are used but during the purification the enzymes will denature. Hence the immobilization technique makes the enzymes stable. The immobilized enzymes are used for the production of syrups. Immobilized beta-galactosidase used for lactose hydrolysis in whey for the production of bakers yeast. The enzyme is linked to porous silica matrix through covalent linkage. This method is not preferably used due to its cost and the other technique developed by Valio in 1980, the enzyme galactosidase was linked to resin (food grade) through cross linking. This method was used for the various purposes such as confectionaries and icecreams [54].

Biodiesel Production:

Biodiesel is monoalkyl esters of long chain fatty acids. Biodiesel is produced through triglycerides (vegetable oil, animal fat) with esterification of alcohol (methanol, ethanol) in the presence of the catalyst. The production of catalyst is a drawback of high energy requirements, recovery of glycerol and side reaction which may affect the pollution. Hence the biological production of liquid fuel with lipases nowadays has a great consideration with a rapid improvement [55]. Lipase catalyses the reaction with less energy requirements and mild conditions required. But the production of lipase is of high cost, hence the immobilization of lipase which results in repeated use and stability [56, 57]. The immobilization of lipase includes several methods entrapment, encapsulation, cross linking, adsorption and covalent bonding. Adsortion method of immobilization is widely used in recent years when compared to covalent bond, entrapment and cross linking [58]. In the biological production of biodiesel the methanol inactivates the the lipase, hence the immobilization method is an advantage for the biodiesel production [59]. The low cost of lipase, candida sp as origin is of more industrial use [60]. The nanostructured carriers are with high porosity [61], natural material activated carbon [62], celite, zeolite [63]. The carriers for lipase immobilization by covalent attachment of olive pomace [64], resins [65], Polyurethane foam [66], chitosan [67], silica [68] and magnetic nanostructures [69], When compared to the natural support material chitosan is used for enzyme binding, the immobilized lipase retains its stability for 10 cycles of pomace oil, esterification, while maintaining 80% residual activity [70].

Wastewater Treatment:

The increasing consumption of fresh water and water bodies are mixed up with polluted industrial waste water and the waste water treatments are necessary at present. The sources of dye effluents are textile industry, paper industry, leather industry and the effluents are rich in dye colourants. These effluents are threat to the environment and even in low concentration it is carcinogenic. Nowadays enzymes are used to degrade the dye stuffs. The enzymes used in the wastewater treatments are preoxidases, laccase, azo reductases. These enzymes due to harsh conditions like extreme temperature, low or high pH and high ionic strength may

lose its activity; to overcome this problem immobilized enzymes are used. The Horse radish Peroxidases are entrapped in calcium alginate beads, this method is still in lab scale research [71]. The immobilized laccase enzyme has the ability to degrade dyes anthracinoid dye, Lancet blue and Ponceau red [72]. Adsorption method is widely used because of its easy regeneration. During the covalent method of immobilization the conformational change in the enzyme occurs which will affect the activity of the enzyme [73]. In Single Enzyme Nanoparticle, the enzyme is protected by a nanometer thick substance as it provides the large surface area. SEN has the ability to retain its activity during the extreme conditions. SEN is also used in the removal of heavy metals from the waste water [74].

Lipase has the ability to hydrolyse oil and fats to long chain fatty acid and glycerol. The immobilized lipase is of high interest for the hydrolysis of oils and fats for treating the waste water from the food industry. The drawback of the conventional treatment methods is slow biodegradability, oil and fats are absorbed on the surface of sludge [75]. Researchers are now focusing on the treatment with immobilized lipase. Lipase immobilized on the sol gel / calcium alginate with the size of 82µm, immobilized lipase. Immobilized lipase operated for 100 days in continuous sludge without any problem, does not produced foam in the reactor [76].

Textile Industry:

The enzymes derived from microbial origin are of great interest in textile industry. The enzymes such as cellulase, amylase, liccase, pectinase, cutinase etc and these are used for various textile applications such as scouring,biopolishing, desizing, denim finishing, treating wools etc. Among these enzymes cellulase has been widely used from the older period to till now. The textile industries now turned to enzyme process instead of using harsh chemical which affects the pollution and cause damage to the fabrics [77]. The processing of fabrics with enzymes requires high temperatures and increased pH, the free enzymes does not able to withstand the extreme conditions. Hence, enzyme immobilization for this process able to withstand at extreme and able to maintains its activity for more than 5-6 cycles. PolyMethyl Methacrylateis linked with cellulase covalently. In this method the nanoparticle is synthesized with cellulase as core particle [78-80]. Endoglucanase is a component of Cellulase enzyme, Endoglucanase is microencapsulated with Arabic Gum is a natural polymer with the biodegradable property is used as a matrix for encapsulation of endoglucanase. Encapsulation of endoglucanase prevented it to retain its activity in the presence of detergents [81].

Detergent Industry:

The detergent industry also employs enzymes for removal of stains. The enzymes used in detergent industry are protease which is used to remove the stains of blood, egg, grass and human sweat. Amylase used to remove the starch based stains like potatoes, gravies, chocolate. Lipase used to remove the stains of oil and fats and also used to remove the stains in cuffs and collars. Cellulase is used for cotton based fabrics in order to improve softening, colour brightening and to remove soil stains [82, 83]. Nowadays Biotech cleaning agents are widely used in the detergent industries. When compared to synthetic detergents the biobased detergents have good cleaning property [84].

The enzymes based detergents can be used in low quantity when compared to the synthetic detergent, it has increased biodegradability, does not affect the environment works well in low temperature, and these are the advantages of enzymes in detergent industry. The immobilized enzymes are also used in immobilized enzymes. Proteases hydrolyse the proteins, and protease cannot be used for keratin based fabric wool and silk which cause adverse damage to the garment. So protease directly cannot be used for wool and silk garments, protease loses its stability in the presence of surfactants and oxidizing agents, hence protease is immobilized by covalently linking with Eudragit S-100 using carbodiimide coupling [85]. The immobilized protease treated with wool for 72hrs with 100U at 40^{o}C the free enzyme was degraded the wool but the immobilized enzyme retained 76% of the tensile strength of wool [86]. Protease also immobilized by entrapment method with polyacryamide gel, the enzyme retained its activity for about 6 cycles with incubation time of 20 min at 55 ^{o}C for each cycles, by this immobilization method, protease activity is retained for about 83%of the initial activity after six cycles [87]. The lipase immobilization is carried in order to prevent lipase from protease action and surfactant inhibition lipase is immobilized on acrylamine glass beads coated with zirconia with the size of 55nm. The Maximum activity was found for immobilized lipase at the pH of 6.5 and for free enzyme it is 7.5 [88].

Properties of Immobilized Enzymes:

The stability of the immobilized enzymes is based on the temperature and time. The activity of the enzyme is retained throughout series of cycles. Due to immobilization, the properties of enzymes will be altered such as catalytic activity with respect to the support matrix. The change in the enzyme properties in the immobilized enzyme is due to the enzyme and the substrate reacts in the microenvironment which is different from the enzyme substrate reaction in the bulk solution environment. The change is also due to the change in the three dimensional conformation of the protein when linked with the support matrix. These conformational changes are to a lesser extent and these changes occur in the limited enzyme systems [89].
Enzyme immobilization improves the operational stability and is also due to the increased enzyme loading

which causes the controlled diffusion. The stabilization as a result of, number of bonds formed between the enzyme and the support matrix. When the immobilized enzyme acts on the macromolecular substrates, active site of the enzymes does not able to access with the substrates, hence the enzyme loses its activity [90].

Conclusion

Enzyme immobilization is widely exploited technique in various industries food industry, pharmaceutical industry, bioremediation, detergent industry, textile industry etc. This method is used due to its technical and economical advantage. Large number of enzymes have been immobilized and used in various large scale processes. This Stabilization method can lower the cost of the enzymes. Enzyme immobilization provides operational stability to enzymes.

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