

Enzyme Immobilization: An Overview on Nanoparticles as Immobilization Matrix

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Abstract

Immobilization process is to optimize the operational performance of an enzyme for industrial applications. So far different matrices have been described in the literature to improve the performance of the immobilized enzymes. With the advent of nanotechnology, the nanomaterials because of their unique physico-chemical properties constitute novel and interesting matrices for enzyme immobilization. The nanomaterials possess ideal characteristics to equilibrate principal factors which determine biocatalysts efficiency, including specific surface area, mass transfer resistance and effective enzyme loading. This review presents the current scenario and techniques in enzyme immobilization. An overview of the main methods used to combine proteins/enzymes with nanoparticles is given in the study. The advantages and disadvantages of nanoparticles as immobilization matrix are also discussed.

Keywords: Immobilization; Nanoparticles; Covalent attachment; Adsorption; Crosslinking

Introduction

Enzymes are catalyst that catalysis many biochemical and chemical reactions. They are universally present in plants and animals. Due to their ease of production, substrate specificity and green chemistry these biocatalyst are widely used in diverse sections. Enzymes have extensive applications in food industries such as baking [1,2], dairy products [3], starch conversion [4] and beverage processing (fruit, vegetable juices, beer and wine) [5]. In textile industries, they have found a special place due to their effect on end products [6]. In industries such as paper and pulp making [7] and detergents [8], the use of enzymes has become a necessary processing strategy. Some of the major class of industries such as health care & pharmaceuticals [9] and chemical [10] manufacturing have been increased due to the catalytic nature of enzymes. Another major application of enzymes is in waste management [11] especially for solid wastes treatment [12] and waste water purification [13-15]. Past few years have marked the significance of enzymes in production of biofuels such as biodiesel, bioethanol, biohydrogen and biogas from biomass conversion [16]. However, all these desirable characteristics of enzymes and their widespread industrial applications are often obstructing by their lack of long-term operational stability, shelf life and by their recovery & reusability. Enzyme immobilization is one of the strategies to overcome these problems.

Enzyme immobilization

Immobilized enzyme was discovered in 1916 [17]. It was demonstrated that activity of invertase enzyme does not get hampered when it is adsorbed on a solid matrix, such as charcoal or an aluminum hydroxide. This aspect led to the development of currently available enzyme immobilization techniques. Initially immobilization techniques used to have very low enzyme loadings, with respect to available surface areas. In late 90s various covalent methods of enzyme

immobilization were developed. While enzyme immobilization has been studied for a number of years, the appearance of recent published research and review papers indicates a continued interest in this area [18,19]. Currently commercial application of immobilized enzyme have been enhanced as they are highly efficient [5,19]. Further, its resistance to various environmental changes such as pH or temperature has been increased during immobilization of enzyme on solid support [20]. Compared to their free forms, immobilized enzymes are generally more stable and easier to handle. In addition, the reaction products are not contaminated with the enzyme which is useful in the food and pharmaceutical industries. Moreover, in the case of proteases, the rate of the autolysis process can be dramatically reduced upon immobilization only, if a multipoint or multisubunit immobilization is achieved, or if a favourable enzyme environment is obtained [21]. Additionally, immobilization also improves many properties of enzymes such as performance in organic solvents, pH tolerance, selectivity, heat stability or the functional stability. Increasing the structural rigidity of the protein and stabilization of multimeric enzymes prevents dissociation-related inactivation [22,23]. The attached enzyme is again ready for the subsequent reactions without the need for repeated, time consuming, and costly extraction and purification procedures [22]. These alterations result from structural changes introduced into the enzyme molecule by the applied immobilization procedure and from the creation of a microenvironment in which the enzyme works, different from the bulk solution [24]. The main objective of enzyme immobilization is to maximize the advantages of enzyme catalysis, which is possible by using a support with low synthesis cost and high binding capacity [25].

The stability of a native enzyme (non-immobilized) is principally determined by its intrinsic structure whereas the stability of an immobilized enzyme is highly dependent on many factors, including the nature of its interaction with the carrier, binding position and number of bonds, the freedom of the conformation change in the matrix, the microenvironment in which the enzyme molecule is located, the chemical and physical structure of the carrier, the properties of the spacer (for example, charged or neutral, hydrophilic or hydrophobic, size, length) linking the enzyme molecules to the

carrier, and the conditions under which the enzyme molecules were immobilized. Hence the stability of the immobilized enzymes with respect to time, temperature and other storage conditions and experimental variables might be expected to either increase or decrease on immobilization [26]. It has been found that many enzymes immobilized by different immobilization techniques have higher activity than the native enzymes. For instance, epoxy hydrolase adsorbed on DEAE-cellulose by ionic bonding was more than twice as active as the native enzyme [27], lipase—lipid complex entrapped in *n*-vinyl-2-pyrrolidone gel matrix was 50fold more active than the native enzyme [28]. Activation by immobilization is, however, often regarded as an additional benefit rather than a rational goal of enzyme immobilization. Activity retention by carrier-bound immobilized enzymes is usually approximately 50%. At high enzyme loading, especially, diffusion limitation might occur as a result of the unequal distribution of the enzyme within a porous carrier, leading to a reduction of apparent activity [29]. The conditions for high activity retention are often marginal, thus often requiring laborious screening of immobilization conditions such as enzyme loading, pH, carrier and binding chemistry [26]. Changes in enzyme properties not necessarily mean improvements, and in some instances a careful and extremely mild immobilization protocol should be used to keep the good properties of the utilized enzyme intact.

Immobilization of enzyme can be carried out by different methods; broadly they are classified as physical and chemical. Physical methods have weak interactions between matrix and enzyme, whereas in chemical methods there is formation of covalent bond between the support and the enzyme. In particular, the development and applications of site selective protein immobilization have undergone significant advances in recent years. It has been noticed that advances in organic chemistry and molecular biology have led to the development of some very powerful, efficient, site-specific, and important applications of anchoring proteins onto supports [30-32]. These have been followed by the development of functional protein microarrays, biosensors, and continuous flow reactor systems [31].

Methods of immobilization

The selection of mode of immobilization is very important to prevent the loss of enzyme activity by not changing the chemical nature or reactive groups in the binding site of enzyme. Considerable knowledge for the nature of the active site of the enzyme will be helpful. On the other hand, active site can be protected by the attachment of protective groups, later on which can be removed without any loss of enzyme activity. In some cases, this protective function can be fulfilled by a substrate or a competitive inhibitor of the enzyme. The most common procedures of enzyme immobilization are adsorption, covalent coupling, entrapment and cross-linking [18]. Figure 1 gives the diagrammatic representation of the various methods of immobilization.

Although various reviews are published on the immobilization methods which give the detailed methodology, protocol of each method and also its advantages and disadvantages. A brief discussion of each method is summarized below.

Adsorption: Adsorption of enzymes onto insoluble supports is a very old and simple method which has wide application and high capability enzyme loading relative to other immobilization methods. Enzymes can be immobilized by simply mixing the enzymes with a suitable adsorbent, under appropriate conditions of pH and ionic strength. After washing off loosely bound and unbound enzyme, the

immobilized enzyme is obtained in a directly usable form. Adsorption process is based on vander Waal forces, ionic and hydrogen bonding as well as hydrophobic interactions, which are very weak forces, but in large number, impart sufficient binding strength.

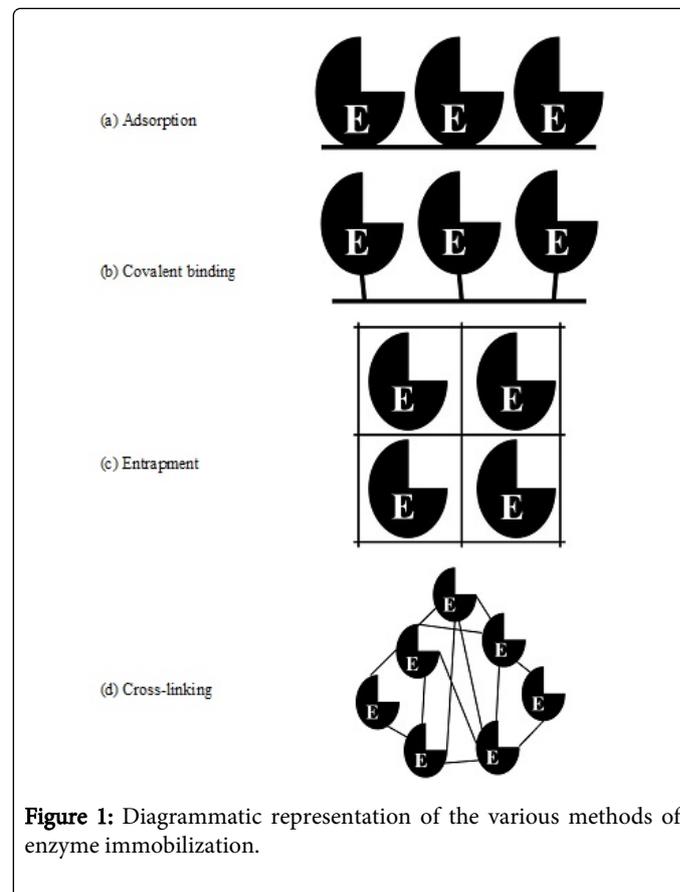


Figure 1: Diagrammatic representation of the various methods of enzyme immobilization.

Adsorbed enzymes can be protected from agglomeration, proteolysis and interaction with hydrophobic interfaces [33]. The choice of adsorbent particularly depends upon minimizing the leakage of used enzyme. In order to prevent chemical modification and damage to enzyme, the existing surface properties of enzymes and support are need to be considered Care must be taken that the binding forces are not weakened during use of unusual changes in pH or ionic strength. The adsorption through physical method generally leads to major changes in the protein microenvironment, and typically involves multipoint protein adsorption between a single protein molecule and a number of binding sites on the immobilization surface [34]. The main disadvantage of this method is that the enzyme is easily desorbed by factors like temperature fluctuations, changes in substrate and ionic concentrations [35].

Covalent binding: Covalent immobilization involves the formation of covalent bonds between the enzyme and the support matrix. The functional groups present in the enzymes get linked to support matrix as these functional group are not responsible for the catalytic activity. The binding reaction must be performed under conditions that do not cause loss of enzymatic activity, and the active site of the enzyme must be unaffected by the reagents used. Covalent association of enzymes to supports occurs owing to their side chain amino acids like arginine, aspartic acid, histidine and degree of reactivity based on different functional groups like imidazole, indolyl, phenolic hydroxyl, etc. [36].

Peptide-modified surfaces when used for enzyme linkage results in higher specific activity and stability with controlled protein orientation [37]. Sometimes functional groups on the support material are activated by certain reagents and enzyme is then coupled to the support material via covalent linkage. Cyanogen bromide (CNBr)-agarose and CNBr-activated-Sepharose containing carbohydrate moiety and glutaraldehyde as a spacer arm have imparted thermal stability to covalently bound enzymes [38,39]. The connection between the carrier and enzyme can be achieved either by direct linkage between the components or via an intercalated link of differing length, which is called spacer. The spacer molecule gives a greater degree of mobility to the coupled biocatalyst so that its activity can be enhanced when compared to that of direct coupled biocatalyst.

Entrapment: It is defined as the restricted movement of enzymes in a porous gel, yet keeping them as free molecules in solution. Entrapment of enzymes within gels or fibers is a convenient method for use in processes involving low molecular weight substrates and products. However, the difficulty which large molecules have in approaching the catalytic sites of entrapped enzymes precludes the use of entrapped enzymes with high molecular weight substrates. The entrapment process may be a purely physical caging or involve covalent binding. Enzymes have been entrapped in natural polymers like agar, agarose and gelatine through thermo reverse polymerization, but in alginate and carrageenan by ionotropic gelation [40]. A number of synthetic polymers like polyvinylalcohol hydrogel [41], polyacrylamide [42] have also been investigated.

Cross-linking: This method involves attachment of biocatalysts to each other by bi- or multifunctional reagents or ligands [40]. In this way, very high molecular weight typically insoluble aggregates are formed. Cross-linking is a relatively simple process. It is not a preferred method of immobilization as it does not use any support matrix. So they are usually gelatinous and not particularly firm. Since it involves a bond of the covalent kind, biocatalyst immobilized in this way frequently undergoes changes in conformation with a resultant loss of activity. Still it finds good use in combination with other support dependent immobilization technologies, namely to minimize leakage of enzymes already immobilized by adsorption. The most commonly used bifunctional agent for cross-linking is glutaraldehyde. The reactive aldehyde groups at the two ends of glutaraldehyde react with free amino groups of enzymes through a base reaction and have been extensively used in view of its low cost, high efficiency, and stability. The enzymes or the cells have been normally cross-linked in the presence of an inert protein like gelatine, albumin, and collagen and can be applied to either enzymes or cells. The main disadvantages are the undesirable activity losses that can arise from the participation of catalytic groups in the interactions responsible for the immobilization. The cross-linking reaction is not easily controlled and so it is very difficult to obtain large enzyme aggregates with high activity retention. The gelatinous physical nature of the immobilized enzyme preparations is a great limitation in many applications. The more recently developed cross-linked enzyme aggregates (CLEAs) are produced by simple precipitation of the enzyme from aqueous solution, as physical aggregates of protein molecules, by the addition of salts, or water miscible organic solvents or non-ionic polymers [43]. These physical aggregates are held together by non-covalent bonding without perturbation of their tertiary structure that is without denaturation. Subsequent cross-linking of these physical aggregates renders them permanently insoluble while maintaining their pre-organized superstructure and hence their catalytic activity. This discovery led to the development of a new family of immobilized

enzymes: CLEAs. This type of immobilized enzyme is very effective biocatalysts as they can be produced by inexpensive and effective method. CLEAs can readily be reused and exhibit satisfactory stability and performance for selected applications. The methodology is applicable to essentially any enzyme, including cofactor dependent oxidoreductases [44].

Although the basic methods of enzyme immobilization can be categorized into a few different methods as mentioned above, hundreds of variations, based on combinations of these original methods, have been developed [40,45,46]. Correspondingly, many carriers of different physical and chemical nature or different occurrences have been designed for a variety of bio-immobilizations and bio-separations [40,47].

Choice of support for immobilization

The characteristics of the matrix are important in determining the performance of the immobilized enzyme system. Ideal support properties include physical resistance to compression, hydrophilicity, inertness toward enzymes ease of derivatization, biocompatibility, resistance to microbial attack, and availability at low cost [48]. Several natural polymer materials like cellulose, alginate, chitin, collagen, carrageenan, chitosan, starch, sepharose, pectin, and other natural polymer materials are commonly used as support materials [40]. Besides, natural polymers various synthetic polymeric materials are also used as support as they possess good mechanical stability, moreover they can be modified easily [49,50]. A variety of inorganic supports are also used for the immobilization of enzymes, e.g., alumina, silica, zeolites, and mesoporous silicas [39,40,51]. Silica-based supports are the most suitable matrices for enzyme immobilization in industrial manufacturing of enzyme-processed products [39,52], as well as for research purposes [53]. Carriers which have large surface area always do a great help to obtain good immobilization efficiency.

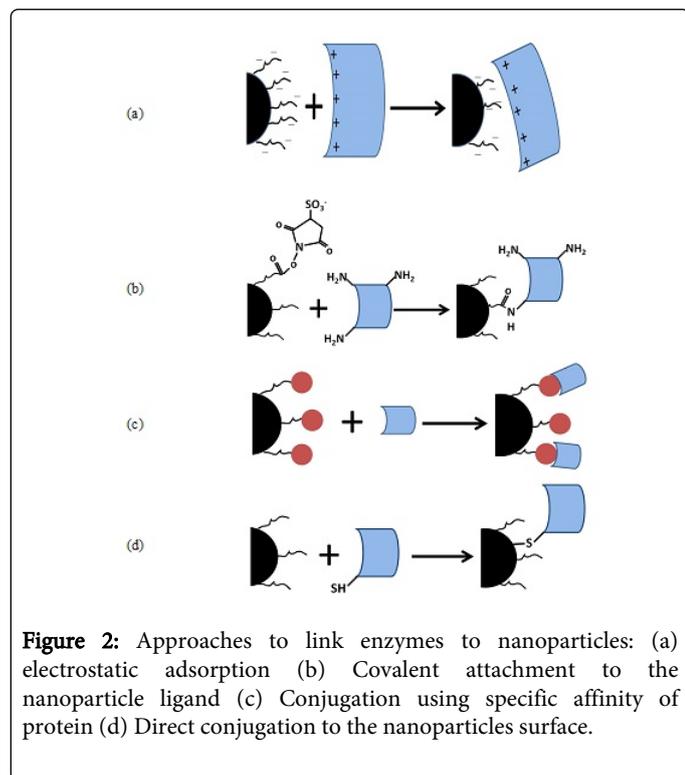
Nanoparticles as immobilization matrix

Nanoparticles act as very efficient support materials for enzyme immobilization, because of their ideal characteristics for balancing the key factors that determine biocatalysts efficiency, including specific surface area, mass transfer resistance, and effective enzyme loading [54-57]. Diffusion problem is more relevant when we are dealing with the macromolecular substrates, for such systems the nanoparticles are the ideal candidates [58]. Moreover, the enzyme bound nanoparticles show Brownian movement, when dispersed in aqueous solutions showing that the enzymatic activities are comparatively better than that of the unbound enzyme [55]. In addition, magnetic nanoparticles possess additional advantage, can be separated easily using an external magnetic field. Studies have shown that immobilization of enzymes to the nanoparticles can reduce protein unfolding and can improve stability and performance [55]. Various reviews on immobilization of enzymes on different types of nanoparticles (metal nanoparticles, metal oxide nanoparticles, magnetic nanoparticles, porous and polymeric nanoparticles) have been published earlier [55,56,59,60]. A few examples of nanoimmobilized enzymes are cited in this review.

Enzymatic immobilization on Au and Ag nanoparticles have been studied using either as whole cells or isolated enzymes, which include lysozyme [61], glucose oxidase [62], aminopeptidase [63], as well as alcohol dehydrogenase [64]. Cruz et al. [65] reported the Immobilization of enzymes *S. Carlsberg* and *Candida antarctica* lipase B (CALB) on fumed silica nanoparticles for applications in

nonaqueous media and they observed catalytic activities were remarkably high. Won et al. [66] immobilized acetylcholinesterase onto magnetic glasses based on iron oxide/silica, for paraoxon sensing. Ganesana et al. [67] performed the immobilization of acetylcholinesterase on nickel nanoparticles and obtained a highly sensitive detection method for organophosphate pesticides. Uygun et al. [68] employed magnetic poly (2-hydroxyethyl methacrylate-N-methacryloyl-(l)-phenylalanine) to immobilize α -amylase. They reported a substrate affinity increases upon enzyme immobilization and showed that a specific activity of 85% was maintained after 10 reuses. Khoshnevisan et al. [69] immobilized cellulase on magnetic nanoparticles obtaining a smaller activity than for the free enzyme, but at 80°C the immobilized enzyme showed slightly greater activity. Lee et al. [70] used amino-functionalized silica-coated magnetic nanoparticles to immobilize trypsin. They applied this system to a pressure-assisted digestion for proteome analysis. It was observed for each of the experiments in which the magnetic nanoparticles were employed an increased number of protein identification in comparison with the experiment with free trypsin. Qiu et al. [71], reported the construction of glucose biosensor using the amino-functionalized Fe₃O₄@SiO₂ nanoparticles covalently bond to ferrocene monocarboxylic acid as the building block. The biosensor reached 95% of the steady-state current within 10 s after the addition of glucose. Recently in our lab we have reported the immobilization of enzymes (Peroxidase, cellulase, trypsin and alpha amylase) on TiO₂ nanoparticles. The immobilized enzymes show higher activity than free enzymes. It also showed enhanced thermal stability compared to its soluble counterpart at higher temperature [72-75].

All the advantages of immobilized enzymes on micron-sized particles are inherited when nanomaterials are used as solid supports. Broadly there are four main approaches to link a protein or enzyme to the nanoparticles as shown in Figure 2.



Electrostatic adsorption: The most widely used linkage approach consists of electrostatic adsorption (Figure 2a). This is the simplest approach and is already used routinely as an electron dense marker in histology [76]. The interaction between the nanoparticle and protein may be modulated by the pH or charge screening by controlling the ionic strength of the medium.

Covalent attachment to the surface modified nanoparticles: Another general method for nanoparticle–protein conjugation is covalently linking a protein to the nanoparticle ligand (Figure 2b). This approach has been greatly advanced by extreme control over the surface chemistry of the nanoparticles. For example, a variety of organic functional groups can be introduced to the surface using mild conditions [77]. The popular labeling chemistry utilizes the covalent binding of primary amines with sulfo-NHS esters or R-COOH groups via reaction with EDC [77]. Nanoparticles labeled with NHS esters can react to form covalent bonds with the primary amine of lysine on a protein. In addition, nanoparticles coated with maleimide groups can react with the thiol of cysteine on a protein. Oxide nanoparticles (TiO iron oxide, Copper oxide, silver and gold oxide) can be easily modified by Silanization yielding a modified surface exhibiting amino groups, which can be used as adsorbent or as coupling sites for linking various proteins.

Conjugation using specific affinity of protein: Nanoparticle–protein conjugation can also be achieved by using specific labeling strategies (Figure 2c). Example Streptavidin coated nanoparticles can selectively bind biotin-labeled proteins and antibody coated nanoparticles selectively bind to the specific protein [78].

Direct conjugation to the nanoparticles surface: A direct reaction of a chemical group on the protein without the use of a linker is usually desired if the particle is used as a biosensor where FRET or electron transfer is used (Figure 2d). For Au and Ag nanoparticles, this can be achieved by the Au-thiol or Ag- thiol chemistry where a protein with a cysteine covalently bonds to an Au or Ag nanoparticle. The conjugation requires incubation of the protein and the nanoparticle together as the Au-S or Ag-S bond is strongly favored. Similarly, for sulphur containing nanoparticles such as ZnS/CdSe, cysteine can directly form a disulfide bridge with the surface S atom. Direct linkages can also be achieved by His tags, which can attach directly to Zn, Ni, Cu, Co, Fe, Mn atoms.

Advantages	Disadvantages
Mass transfer resistance	Cost of fabrication process
Effective enzyme loading	Large scale application
High surface area	Separation of the reaction medium
	(except magnetic nanoparticles)
High mechanical strength	
Diffusional problems minimization	

Table 1: Advantages and disadvantages of using nanoparticles for enzyme immobilization.

Some important new consequences arise when the size of the carrier approaches nanodimensions. Mostly, these all work out in the favour of using nanosized materials. Table 1 Summarizes the advantages and

disadvantages of the use of nanoparticles for enzyme immobilization, considering general aspects.

Present studies shows that different types of nanomaterial are available for enzyme immobilization for examples carbon nanotubes (CNTs), nanoparticles, magnetic nanoparticles, mesoporous media, nanofibers, nanocomposites, nanorods and sol-gel materials containing nanometer-size particles and single-enzyme nanoparticles [79]. However, the major problem for their application is their high cost and complex supports preparation [80]. Strategies or protocols for synthesizing the nanoparticles should be developed which are low cost, ecofriendly and can be used for large scale synthesis.

Selected applications of immobilized enzymes

Industrial applications of immobilized enzymes include laboratory-scale organic synthesis and analytical and medical applications [48,81]. Furthermore, since enzymes are able to catalyze reactions not only in aqueous solutions but also inorganic media, immobilized enzymes can catalyze organic synthesis [82]. DiCosimo et al. [5] mentioned many uses for immobilized enzymes, as high-fructose corn syrup production, pectin hydrolysis, debittering of fruit juices, interesterification of food, fats and oils, biodiesel production, carbon dioxide capture and, in most cases, it can be extended to “nano” sizes. Table 2 shows few applications of nanoimmobilized enzymes.

Enzyme	Nanoparticle	Application	References
β-Glucosidase (BGL) from <i>Aspergillus niger</i>	Iron oxide	Biofuel production	[83]
	nanoparticles		
Superoxide dismutase (SOD)	Nano Fe ₃ O ₄ coated	Biosensors	[84]
	on a gold electrode surface		
α-Amylase	Silica nanoparticles	Formulation of detergent for enhancing removal of starch soils	[85]
Trypsin	Nanodiamond prepared by detonation (dND)	Proteolysis	[86]
Lysozyme	Chitosan nanofibers	Antibacterial	[87]
<i>Mucor javanicus</i> lipase	Nano-sized magnetite	Solvent-free synthesis of 3-diacylglycerols	[88]
Lipases from <i>C. rugosa</i> and <i>Pseudomonas cepacia</i>	Zirconia nanoparticles	Resolution of (R,S)-ibuprofen and (R,S)-1-phenylethanol, respectively	[89]
Horseradish peroxidase (HRP)	Magnetite silica nanoparticles	Immunoassays	[90]
Alcohol dehydrogenase from <i>T. brockii</i> (TbADH)	Gold and silver	Alcohol synthesis	[91]
	nanoparticles		
Cholesterol oxidase	Fe ₃ O ₄ nanoparticles	Analysis of total cholesterol in serum	[92]
Haloalkane dehalogenase	Silica coated iron oxide nanoparticle	Production of fusion proteins containing dehalogenase sequences	[93]
Laccase	Chitosan-magnetic nanoparticle	Bioremediation of environmental pollutants	[94]
Keratinase	Fe ₃ O ₄ nanoparticles	Synthesis of keratin	[95]
α-Amylase	Cellulose-coated magnetite nanoparticles	Starch degradation	[96]
B-Galactosidase	Con A layered ZnO nanoparticles	Lactose hydrolysis	[97]
Lipase	Fe ₃ O ₄ nanoparticles	Hydrolysis of pNPP	[98]
Glucose oxidase	Thiolated gold nanoparticle	Estimation of glucose level up to 300 mg/ml	[99]
Lipase	Polystyrene nanoparticle	Aminolysis, esterification,	[100]

		trans-esterification	
α -Chymotrypsin	Polystyrene nanoparticles	Proteolysis (cleaves peptide amide bonds)	[101]
Diastase	Silica coated nickel nanoparticle	Starch hydrolysis	[102]
Bitter Gourd Peroxidase (BGP)	TiO ₂ nanoparticles	Phenol and dye removal	[73]
Cellulase	TiO ₂ nanoparticles	Hydrolysis of Carboxy methyl cellulose	[75]
Alpha amylase	TiO ₂ nanoparticles	Starch hydrolysis	[72]
Alpha amylase	TiO ₂ nanoparticles	Starch hydrolysis and refolding	[74]
Trypsin	TiO ₂ nanoparticles	refolding	[74]
Alpha amylase	Silver nanoparticles	Starch hydrolysis	[103]

Table 2: Enzymes immobilized on nanoparticles and their biotechnological applications.

Conclusions

Immobilization process has been used for enhancing enzyme activity and stability in aqueous and non-aqueous media. Selecting and designing the support matrix are important in enzyme immobilization. Recently, the use of nanoparticles has emerged as a versatile tool for generating excellent supports for enzyme stabilization due to their small size and large surface area. It has been observed that the stability and activity of enzymes increases when immobilized on such materials. The nanomaterials are key components in the future market of high technology. Nanoparticles strongly influence the mechanical properties of the material like stiffness and elasticity and provide biocompatible environments for enzyme immobilization.

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