Review Article

# Use of Nanomaterials for the Immobilization of Industrially Important Enzymes

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## Abstract

Immobilization enables enzymes to be held in place so that they can be easily separated from the product when needed and can be used again. Conventional methods of immobilization include adsorption, encapsulation, entrapment, cross-linking and covalent binding. However, conventional methods have several drawbacks including reduced stability, loss of biomolecules, less enzyme loading or activity and limited diffusion. The aim of this study is the evaluation of importance of nanomaterials for the immobilization of industrially important enzymes. Nano materials are now in trend for the immobilization of different enzymes due to their physi-chemical properties. Gold nanoparticles, silver nanoparticles, nano diamonds, graphene, carbon nanotubes and others are used for immobilization. Among covalent and non-covalent immobilization of enzymes involving both single and multiwalled carbon nanotubes, non-covalent immobilization with functionalized carbon nanotubes is superior. Therefore, enzymes immobilized with nanomaterials possess greater stability, retention of catalytic activi-

ty and reusability of enzymes.

**Keywords:** Catalytic activity; Covalent immobilization; Carbon nanotubes; Nanomaterials; Hydrolases

## 1. Introduction

Enzymes are the architecture of proteins which act as catalyst molecules that carry out specific biochemical reactions in the body. Enzymes are extremely well-organized catalysts explored for commercial catalytic properties because of their numerous benefits [1]. Many enzymes are being used in the different industries, providing a huge number of benefits. Some of the most important industrial enzymes include pectinase, hydrolyses, cellulose, lipase, phytases and lignocelluloses. Pectin is the very complex polysaccharide existing in the cell wall of the plant while the pectinases are the depolymerizing enzyme that can be distributed into two classes; one is the hydrolyses and other one is lyases. A huge number of pectinases are present in the plants and the microorganisms classes and have dynamic part in the extension of cell wall and in the softening of the plant tissues [2]. Acidic pectinases have their role in juice clarification while alkalophilic provide benefits in dissolving of plant materials [3]. Phytase is the most important enzyme in the food industry and is the major storage form of the phosphorous in the grains. Phytase acts on the phytate and release organic phosphorous for the animals to reduce dependent on inorganic phosphorous supplements and to provide nutritional benefits [4]. Lignocellulose immobilization is used for production of ethanol industrially. The production process is only applied at domestic scale due to technical problems which can be resolved by using dry mass (20%) [5]. Lipases are the natural enzyme that catalyze the hydrolysis of triglycerides while in the non-aqueous state they catalyze the reverse reactions (esterification reactions) and in this way are used in situ lipid metabolism and ex situ multidimensional industrial application [6].

The term immobilized enzymes states to restrict enzyme physically or confine enzyme in a specific state with the holding of the catalytic property that can be utilized repeatedly. As it is better to remove the presence of unimportant complexes or molecules in the final products the possibility to abolish the enzyme is very substantial in the food industry. Till to date, industrial enzymes have been immobilized on numerous supports that may include nylon ion exchange resin, silk, and chitin [2]. Enzyme immobilization is the ultimate method for the expansion of bioreactors and biosensors. Besides easily separation from the product, immobilized enzymes have the benefit of heat and functioning constancy in the presences of dangerous levels of pH, temperature and organic solvent [7]. They are, thus, the accurate applicant to use industry for large scale applications. Commercial applications of immobilized enzymes can result in both enhanced product excellence and lesser treating price [7]. The grape skin cell wall establishes a blockade against flow of polyphenols that can be removed by hydrolysis of their fundamental polysaccharides (pectin, hemicellulose, and cellulose), a process that can be enabled by maceration enzymes So the seed extracted in this way is examined for its ability as a cold-active acidic enzyme source [8].

The methods used for the immobilization include 1) Adsorption which includes Van der Waals forces, ionic bond and hydrogen bonding interactions [9]. This method is done by mixing the enzyme(s) and a support material with each other in adsorption properties, at optimum pH, ionic strength [10]. 2) The pore size of a gel lattice is controlled to ensure that the structure become tight enough to prevent loose of enzyme or cells, it also allow free movement of the substrate and product [9]. 3) Encapsulation of enzymes as well as cells can be accomplished by wrapping the biological components inside different forms of semi permeable membranes [11]. Entrapment in that the enzymes/ cells are free in movements but limited in space [9]. 4) Cross linking is the method of immobilization depend only on enzyme and it is support- free as it done by joining the enzyme (or the cells) to each other to prepare a large, three-dimensional complex structure [9]. 5) Covalent Bonding is formed between the functional groups present on the surface of carrier and the surface functional groups of the enzyme [12].

Nano materials are now in trend for the immobilization of different enzymes. They increase surface area to volume ratio [13] which increase the stability of the immobilized enzyme and increase enzymatic performance. Gold nanoparticles [14, 15], silver nanoparticles, graphene, carbon nanotube, carbon Nano fiber [16], magnetic bio nanoparticles [17], porous and polymeric nanoparticles and carbon nano composites [18] are being used in immobilization technique. Physicochemical properties of nanomaterials make useful matrices for immobilization [19]. Carbon nanotubes (CNTs) consist of graphene sheets rolled up into hollow cylindrical shape having diameter less than 100nm with length up to few micrometers [20]. CNTs are preferred nanomaterial for immobilizing enzymes due to their chemical inertness [21], exceptional structure, biocompatibility, thermal properties, mechanical properties [22], large surface area and electrical and magnetic properties [23]. Thus, enable greater loading density of enzymes with enhanced stabilization and retention of their catalytic activity [24]. This led towards the development of biosensors, biofuel cells, drug carriers [25] and industrial biocatalysts [26]. Single walled carbon nanotube (SWNT) provide better surface area and multi walled carbon nanotube nanotubes (MWNT) are economically important. Non-covalent and covalent immobilization have been adopted for immobilization [20].

Covalent immobilization gives strong attach-ment however enzyme structure may become denatured [27]. Direct conjugation of R-chymosin and soyabean peroxidase onto SWNT reduced their activity. So, non-covalent functionalization of CNTs [28] with polymeric, organic and biological molecules provide biocompatible nanotube composites for immobilization [27]. Immobilized enzymes are used because of the reason that they remain active for longer time periods even at high temperatures [29]. Lactase enzymes are used milk industry as they hydrolyze lactose which is the major component of milk. Pectinolytic enzymes are used frequently in juice and wine industry [30]. They are used to improve the texture and quality of paper [31]. Lipases are used in the synthesis and hydrolysis of ester bond. Xylanases, amylases and cellulases are used for the degradation of biomass [32].

## 2. Conventional Methods for Immobilization of Enzymes

Enzyme immobilization is in practice since 1916, Nelson and Griffin discovered that Invertase l has the ability to hydrolyze the sucrose, when it is absorbed to charcoal. Grubhofer and Schelth introduced the ability of enzyme to react even after immobilization. The repeated assay can be done with the immobilized enzyme [12]. In the early Phase, 1916-1940, Glass, Alumina. Hydrophobic compound coated glass were used for immobilization of enzymes. Underdeveloped Phase was 1950 in which Non-specific physical adsorption of enzymes on solid carriers was used along with amylase to adsorb in carbon. Developing Phase 1960, involved entrapment of enzymes. The fully developed era includes work on immobilization of enzymes including the nanotechnology and nano-particles [11]. Immobilized enzymes are more likely to be stable than those enzymes which are in dissolved form. However, there are some draw backs including retardation of enzyme activity, change in kinetic properties, and diffusion or mass transfer limitations. Enzyme immobilization is the technique which is specifically designed to retarder its movement or motility [33]. Immobilization reduces the cost of assay and the enzyme can be reused .It is also very simple and it can be attained through ultrafiltration technique [12]. There are the conventional methods mentioned below Adsorption is the easiest technique for immobilization and here the interaction is totally opposite between carrier and enzyme. Weak forces are formed that are electro-static, for example Van der Waals forces, ionic bond and hydrogen bonding interactions, hydrophobic bonding could be significant, but these forces are very weak but large in number to cover-up all the flaws.

Enzyme(s) and a support material were mixed in adsorption properties, optimum pH, ionic strength, etc. After that the immobilized enzyme was collected and washed to remove unbound enzymes. The enzyme was perfectly immobilized by hat method [10]. Advantages of that method were observed as: it caused little or no damage. The carrier or enzyme/ cells undergoes no change. It's also inexpensive, Easy, and it was found reversible. Disadvantages are: It caused leakage of enzyme/cells from the support the isolation of end- product was found very difficult. It caused nonspecific binding. Nonspecific binding may also lead to dispersal restriction and reaction kinetic problem. Immobilization method of covalent binding holds the creation of a covalent bond or bonds, robust bond, between the enzyme and a carrier. This covalent bond is formed amid the functional groups which are present on the surface of carrier and that of the enzyme. These functional groups are on the surface of enzyme such as amino groups ($NH_2$) of arginine or lysine, carboxylic group (COOH) of glutamic acid or aspartic acid, hydroxyl group (OH) of threonine or serine, and sulfhydryl group (SH) of cysteine [34]. Specific carrier selection is very much affected by many factors.it is mentioned by the research work that hydrophilicity is an important factor for holding up enzyme activity. Polysaccharide polymers are popular materials for enzyme immobilization that are highly hydrophilic. For example, cellulose, starch, spadix, and agarose (sepharose). The sugar remains in these polymers comprise ideal functional groups, hydroxyl groups, for covalent bond formation. Also, hydroxyl groups can produce hydrophilic atmosphere in acquis solution by forming hydrogen bonds. Bead formed supports are used [12].

In entrapment enzymes are blocked in the engineered or regular polymeric systems, it is a porous layer which enables the substrates and the items to pass, yet it holds the catalyst inside the system, the entrapment can be accomplished by the gel, one of the least demanding methods of immobilization is entanglement. As of late, calcium alginate has fascination as an immobilization bolster material. It has been used for immobilization of assortment of cell types, sub-cell organelles, multi-segment frameworks, and [11]. In ionic binding the holding required between the compound and the help material is salt linkages. The idea of this noncovalent immobilization, the procedure will be turned around by changing the temperature extremity and ionic quality conditions. This guideline is like protein-ligand cooperation's standards utilized in chromatography. In affinity binding metal connected chemical immobilization, the metal salts are hastened over the surface of the matrix and it can possibly tie with the nucleophilic bunches on the framework. The precipitation of the metal particle on the transporter can be accomplished by warming. This strategy is straight-forward, and the movement of the immobilized proteins is generally high (30-80%). The transporter and the protein can be isolated by diminishing the pH, henceforth it is a reversible procedure. In the technique of cross linking for immobilization depend just on catalyst and it is sans bolster as it done by joining the compound (or the cells) to one another to set up a huge, three-dimensional complex structure, enzymatic crosslinking typically incorporates development of covalent linkage between the cells by methods for a bi-or multifunctional reagent, for instance glutaraldehyde and toluene diisocyanate. Catalytic ballasts are used for more yield in less time the percentage of ballasts used are 90-99 % [34]. Be that as it may, restricting elements can be utilized in this strategy for living cells and numerous catalysts in view of destructive materials. To limit the nearby issues that can be found as a result of crosslinking of single enzyme type, both egg whites and gelatin have been utilized [35].

## 3. Nanomaterials for Immobilization of Enzymes

In recent searches nanomaterials are now in quick use and are involved in a lot of experiments. There are different types of nanoparticles on the basis of materials used.

### 3.1 Nanometals

By using the MOF (Metal-organic framework) Nano composites were formed. MOFs were formed by a metal ions series like Fe3+, Zr4+ and La3+, these three ions connected with a material that is 2-aminoterephthalate (H2ATA) which formed three MOFs. These MOFs then went through annealing process in a N2 atmosphere and this was done on 550° C. From microstructure and morphological analysis, it was revealed that MOFs original structure retained in this reaction. Then these materials which were derived from the MOF were used for the immobilization [18]. Random movements of the enzymatic molecules that are bounded with the nanoparticles are more stable and the activities of these enzymes are better than the unbounded enzymes. Iron nanoparticles are used for this purpose and the benefit of this bounded iron is that it can be removed easily afterwards by the simple use of electromagnetic radiations [36]. Nanomaterials are being used as matrices for immobilization of many enzymes like lipase (Candida rugosa) enzymes.

SWNT and MWNT are mostly used nanoparticles. Firstly, SWNT were used then MWNT formed which are used more as nano-biocatalysts [19].

Tin dioxide is used as support for the immobilization of the C. rugosa lipase (Nano-SnO2-CRL). On its comparison with the polypropylene (PP-CRL) it was clear that the use of nanomaterial that is tin dioxide increased the efficiency eight times than that of the polypropylene. After one hour of activity nano-based immobilized enzyme retained the 45% activity but polypropylene based immobilization completely inactivated the enzyme. Researchers suggested that the size of the material is need to be optimized for the maximum loading of the tin dioxide [37]. Material of nanoparticles effect the functioning and immobilization of the specific enzyme. Here is the example of Pectate lyase (PL) in which formerly Ca nanoparticles were used in the immobilization procedure but then for the improvement of PL stability Ca nanoparticles were replaced by Calcium Hydroxyapatite and SWNT were used for its entrapment, this replacement was fruitful as it doesn't only stabilize enzyme at low temperature but also at high temperature. As the enzyme is psychrophilic in nature so it was stable only at low temperature but at high temperature it remains stable by maintaining its activity [38].

### 3.2 Gold and silver nanoparticles

Gold and silver nanoparticles are in use for different enzyme immobilization techniques, in these processes either enzyme or whole cell is used, here is the example of one enzyme that is alcohol dehydrogenase [39, 40]. Gold nanoparticles were used in the immobilization of tyrosinase enzyme. Immobilization of enzyme was done by using the solution of gold nanoparticles along with silicate sol-gel matrix and then assembly was done on the surface of ITO electrodes. Aim of using gold nanoparticles was to make the enzyme more stable with better catalytic properties, conductivity, electron transfer ability, optical and electrochemical properties [14].

### 3.3 Nanodiamond

Diamonds and graphene are one of the most stable crystalline structures [41]. Nano diamonds are preferably used in nanobiotechnology as they are biocompatible because of their non-toxic nature and extraordinary cellular uptake. Nano diamonds are used in the immobilization of alcohol dehydrogenase which is obtained from the Saccharomyces cerevisiae. Nano diamonds immobilized under optimum pH were observed which retain 70% activity as compare to the lower efficiency other methods [42].

### 3.4 Nanofibers

Electro spun nano-fibers are recognized as the best support for the immobilization of the enzyme because it gives best surface area to volume ratio, multiple attachment sites and low limitations of mass transfer [43]. Polyaniline which is used for immobilization of L-asparaginase showed best stability at different pH and different temperatures. In comparison with some other methods this material shows stability even after 40 cycles at pH 8.5 and temperature 37◦C [44]. Cellulose nanofibers which are mostly used for different methods are prepared by mechanical or enzymatic methods by using plant fibers [45]. Glucose oxidase is that enzyme, which is immobilized by using porous matrix, which is of polyaniline nanofibers enzyme. This nanofiber matrix helps in immobilizing the enzyme in three steps. 1$^{st}$ step is absorption 2$^{nd}$ one is precipitation, third and the last one process is cross linking (EAPC) [46].

### 3.5 Nanographene

Cellulase immobilization was done on the nano-support made up of magneto-responsive graphene. This type of support is very effective in many bioactive components immobilization [47]. Nano-graphene oxide (GO) is being used for the immobilization of different kinds of enzymes and this is used as a model support for many proteins and important enzymes as it provides great surface functionalization, solubility, larger surface area and rich oxygen. GO based immobilization depends on covalent coupling or it depends on the physical absorption which is non-specific [48, 49].

## 4. Carbon Nanotubes for Immobilization of Enzymes

Nanotube chemistry and method employed for immobilization of enzyme on Carbon nanotube influence activity of CNT-enzyme conjugate [20]. Goh *et al.* merged iron oxide nanoparticles with SWNTs to generate magnetic SWNTs. They immobilized Amylo-glucosidase on magnetic SWNT by covalent immobilization and non-covalent immobilization (physical adsorption). Immobilized enzyme retained its catalytic activity up to 40% upon repeated use, up to many cycles during starch hydrolysis. Separation of nanotube from reaction mixture by magnet detached the enzyme making its reusability possible. Enzyme retained its activity for 1 month at 4º C storage thus making the enzyme cost effective for applying on industrial scale for biofuel production [24]. In another study, immobilization of lipases and esterases on CNTs suggested that curvature of nanotube affect the immobilization yield, structure and catalytic behavior of enzyme. Hydrolases possess high catalytic activity. Covalently immobilized enzymes on amine-functionalized CNTs possess greater catalytic activity and operational stability as compared to physically adsorbed enzymes [50]. There are two ways for the immobilization of industrially important enzymes on carbon nanotubes named as covalent immobilization and non-covalent immobilization.

### 4.1 Covalent immobilization

Covalent immobilization of Organophosphate hydrolase (OPH) on functionalized SWNT and MWNT led towards the development of sensors with high sensitivity and durability. Covalently immo-bilized OPH on SWNT retained higher catalytic activity than OPH immobilized on MWNT [27]. Lipase immobilized covalently on MWNT when subjected to analysis by FTIR spectroscopy and CD, revealed that Lipase-MWNT conjugate show less dependence on temperature than free lipase. Further, immobilized lipase had better stability [51]. Another approach employed controlled placement of enzymes on CNT by using Comb-branched DNA. Foundation DNA strand was covalently attached to MWNT on glassy carbon electrode. In this approach, Comb-Branched DNA was prepared by using Deoxy-ribozyme to bind DNA strand at peculiar location on foundation strand. By altering foundation strand, placement of DNA strands could be adjusted which allowed distance optimization between enzyme and the surface of electrode. Using bioconjugation, Glucose dehydrogenase and alcohol dehydrogenase were bound to comb-branched DNA which resulted in enzyme immobilization on the surface of electrode. Amperometric analysis revealed that length of Foundation strand and distance determine the current response of enzymes in the presence of suitable substrate [52].

### 4.2 Non-covalent immobilization

Non-covalent immobilization is superior approach for enzyme immobilization on CNTs than covalent immobilization due to maintenance of structural confirmation of immobilized enzyme and help in preventing loss of catalytic activity. Direct physical absorption is the most common non-covalent immobilization which involve π-π interactions and hydrophobic interactions between enzyme and nanotube surface [20]. Catalase was adsorbed onto SWNT, oxidized SWNT (O-SWNT) and MWNT. Upon analysis, reduction in catalytic activity was observed most in case of O-SWNT, more in case of SWNT and less in case of MWNT. Fourier transform infrared spectroscopy (FTIR) and circular dichroism (CD) revealed loss in structure of enzyme which was adsorbed onto MWNT than that on SWNT. Increase in number of β sheets was found for Catalase adsorbed onto O-SWNT due to hydrogen bonding between enzyme and nanotube which maintained the enzyme structure and hence function [53]. Laccase enzyme was immobilized onto MWNT and O-MWNT for investigating catalytic activity of immobilized enzyme. Activity was reduced more in case of MWNT and less in O-MWNT. Structure loss was not observed [21].

## 5. Applications of Immobilized Enzymes

### 5.1 Applications of lactase enzyme in dairy industry

B-galactosidase is the enzyme also known as lactase is the most important enzyme used in the dairy industry. This enzyme is used to hydrolyze the lactose which is a disaccharide sugar. This is quite beneficial for the people who are lactose intolerant. Lactose is the major component of the milk products and the people who are deficient of lactase enzyme cannot consume milk products. By, the addition of lactase in milk the sweetness of milk is increased. In this way, more flavor can be added to the food items. Even the byproducts of the food processes can be utilized, and their nutritional value can be increased. For example, whey can be converted to the whey beverages by the addition of lactase [54].

### 5.2 Applications of pectinolytic enzymes

Pectinolytic enzymes are being used worldwide at the industrial scale for the production and clarification of juices and wines. Immobilized enzymes are preferred at the larger scale productions because they remain stable and active for longer time period and at high temperature. Moreover immobilized enzymes can be recovered for reuse [29]. Phyto-therapeutic properties of pectic substances and their modified products are being studied these days to produce nutraceuticals with application in dietary nutrition and pharmacy [30]. Sugarcane bagasse can be used in pulp and paper production [32, 55]. Pectinase pretreatment has potential for improving the efficiency and environmental friendliness of bagasse soda-AQ pulping. The brightness and physical strength properties of the pulp were noticeably improved by the pectinase pretreatment. The properties of the pulp fibers after pretreatment, such as higher fiber length, lower fine length, and higher percent of flexible fiber, would be beneficial to subsequent pulping [31]. Saccharification of agricultural substrates: Pectinases are also being used in biorefineries for hydrolyzing pectin present in pectin-rich agro-industrial wastes [56]. Processing of textile material: Bio-scouring is an eco-friendly method for removal of non-cellulosic impurities from the fiber with the help of enzymes. Improved results are achieved, when pectinase have been used to remove sizing agents from cotton in a

safe and eco-friendly manner, replacing toxic caustic soda [56]. During wastewater treatment especially when water is coming from food industries, to overcome the problem of membrane fouling (MF), biocatalytic membrane reactors with covalently immobilized pectinase were used to develop self-cleaning MF membrane. The biocatalytic membrane with pectinase on its surface gave a 50% higher flux compared to its counterpart inert membrane [57].

### 5.3 Applications of lipases

Lipase enzymes are the most suitable enzymes for catalyzing the biochemical reactions due to their distinguished properties as they are cost effective, easily available and very specific in their action. They are used in pharmaceutical industries and fuel industries as they are involved in wide range of synthesis reactions, like ester bond formation and hydrolysis [58]. Lipases have exclusive property that they can carry out reactions at the edge between aqueous and non-aqueous media. Lipases are extensively used in the formulation of detergents which are used on the daily basis in houses to wash the clothes and dishes [59].

### 5.4 Applications of xylanases, amylases and cellulases

These enzymes are used to hydrolyze the plant biomass. Their potential to be used in energy, fuel and food industries is being studied [32]. These immobilized enzymes are used in the saccharification processes. Cellulases are widely used in food and agricultural biotechnology for cosmetics, detergents, chemicals, pulp and paper synthesis [60].

## 6. Conclusion

The efficacy and stability of a chemical reaction is increased by using the enzymes instead of the conventional methods. The enzyme activity is greatly enhanced by immobilizing them. Conventional methods are not reliable in a sense that they make enzymes less stable and show decreased diffusion. Nano particles have taken excellence in this regard because of their exclusive physiochemical properties. There are covalent and non-covalent immobilization methods using single walled and multi walled carbon nanotubes. Noncovalent immobilization method is superior. Industrialists are more concerned towards the use of immobilized enzymes because at larger production scale, enzymes remain active and stable for longer time period and can be reused. Along with the recent development of immobilized enzymes we will keep on striving to revolutionize this interesting field.

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## Conflicts of Interest

The authors declare no conflict of interest.

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