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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. Nanomaterials are now in trend for the immobilization of different enzymes due to their physicochemical properties. Gold nanoparticles, silver nanoparticles, nanodiamonds, graphene, carbon nanotubes and others are used for immobilization. Among covalent and non-covalent immobilization of enzymes involving single and multi-walled carbon nanotubes, non-covalent immobilization with functionalized carbon nanotubes is superior. Therefore, enzymes immobilized with nanomaterials possess greater stability, retention of catalytic activity and reusability of enzymes.

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

1. INTRODUCTION

Enzymes are the architecture of proteins that 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 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 another one is lyases. A huge number of pectinases are present in the plants and the microorganisms' classes and have a dynamic part in the extension of the cell wall and the softening of the plant tissues [2]. Acidic pectinases have their role in juice clarification while alkalophilic provide benefits in the dissolving of plant materials. Phytase is the most important enzyme in the food industry and is the major storage form of phosphorous in grains. Phytase acts on the phytate and releases organic phosphorous for the animals to reduce dependence on inorganic phosphorous supplements and to provide nutritional benefits [3].

Lignocellulose immobilization is used for the production of ethanol industrially. The production process is only applied at a domestic scale due to technical problems which can be resolved by using dry mass (20%). Lipases are the natural enzyme that catalyzes 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 [4].

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. Until now industrial enzymes have been immobilized on numerous supports that may include nylon ion exchange resin, silk, and chitin enzyme. Immobilization is the ultimate method for the expansion of bioreactors and biosensors. Besides easy separation from the product, immobilized enzymes have the benefit of heat and functioning constancy in the presence of dangerous levels of pH, temperature and organic solvent [5]. They are, thus, promising applicants or candidates for use in the industry. Commercial applications of immobilized enzymes can result in both enhanced product excellence and lesser treating price. The grape skin cell wall establishes a blockade against the 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 [6].

The methods used for the immobilization include the following:

- 1) Adsorption includes Van der Waals forces, ionic bond and hydrogen bonding interactions. This method is done by mixing the enzyme(s) and support material with each other in adsorption properties, at optimum pH, ionic strength [7].
- 2) The pore size of a gel lattice is controlled to ensure that the structure becomes tight enough to prevent loss of enzyme or cells, it also allows free movement of the substrate and product.
- 3) Encapsulation of enzymes as well as cells can be accomplished by wrapping the biological components inside different forms of semi-permeable membranes [8]. Entrapment in that the enzymes/cells are free in movements but limited in space.
- 4) Cross-linking is the method of immobilization that depend only on enzyme and it is support-free as it was done by joining the enzyme (or the cells) to each other to prepare a large, three-dimensional complex structure.
- 5) Covalent bonding is formed between the functional groups present on the surface of the carrier and the surface functional groups of the enzyme [9].

Nanomaterials are now in trend for the immobilization of different enzymes. They increase surface area to volume ratio which increases the stability of the immobilized enzyme and increase enzymatic performance. Gold nanoparticles, silver nanoparticles, graphene, carbon nanotube, carbon nanofiber [10], magnetic bio nanoparticles, porous and polymeric nanoparticles and carbon nanocomposites are being used in immobilization techniques. The physicochemical properties of nanomaterials make useful matrices for immobilization [11].

Carbon nanotubes (CNTs) consist of graphene sheets rolled up into hollow cylindrical shapes having a diameter less than 100 nm with a length up to few micrometres. CNTs are preferred nanomaterial for immobilizing enzymes due to their chemical inertness, exceptional structure, biocompatibility, thermal properties, mechanical properties, large surface area and electrical and magnetic properties [12]. Thus, enable a greater loading density of enzymes with enhanced stabilization and retention of their catalytic activity [13].

This led to the development of biosensors, biofuel cells, drug carriers and industrial biocatalysts. Single-walled carbon nanotubes (SWNT) provide better surface area and multi-walled carbon nanotube nanotubes (MWNT) are economically important. Non-covalent and covalent immobilizations have been adopted for immobilizations. Covalent immobilization gives strong attachment, however enzyme structure may become denatured [14]. Direct conjugation of R-chymosin and soybean peroxidase onto SWNT reduced their activity. So, non-covalent functionalization of CNTs with polymeric, organic and biological molecules provide biocompatible nanotube composites for immobilization [15].

Immobilized enzymes are used because of the reason that they remain active for longer periods even at high temperatures. Lactase enzymes are used in the milk industry as they hydrolyze lactose which is the major component of milk. Pectinolytic enzymes are used frequently in the juice and wine industry. They are used to improve the texture and quality of the paper. Lipases are used in the synthesis and hydrolysis of ester bonds. Xylanases, amylases and cellulases are used for the degradation of biomass [16].

2. CONVENTIONAL METHODS FOR IMMOBILIZATION OF ENZYMES

Enzyme immobilization is in practice since 1916, Nelson and Griffin discovered that Invertase I can hydrolyze the sucrose when it is absorbed into charcoal. Grubhofer and Schelth introduced the ability of the enzyme to react even after immobilization. The repeated assay can be done with the immobilized enzyme [17]. In the early phase, 1916-1940, hydrophobic compound coated glass was used for the immobilization of enzymes. The 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 the entrapment of enzymes. The fully developed era includes work on immobilization of enzymes including nanotechnology and nanoparticles [18].

Immobilized enzymes are more likely to be stable than those enzymes which are in dissolved form. However, there are some drawbacks including retardation of enzyme activity, change in kinetic properties, and diffusion or mass transfer limitations. Enzyme immobilization is the technique that is specifically designed to retarder its movement or motility [19]. Immobilization reduces the cost of assay and the enzyme can be reused. It is also very simple and can be attained through the ultrafiltration technique. There are the conventional methods mentioned below

Adsorption is the easiest technique for immobilization and here the interaction is opposite between carrier and enzyme. Weak forces are formed that are electrostatic, 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.

The 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 the heat method [20]. Advantages of that method were observed as: it caused little or no damage. The carrier or enzyme/cells do not change. It's also inexpensive, easy, and it was found reversible. Disadvantage resulted in 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 problems.

The immobilization method of covalent binding holds the creation of a covalent bond or bonds, a robust bond, between the enzyme and a carrier. This covalent bond is formed amid the functional groups which are present on the surface of the carrier and that of the enzyme. These functional groups are on the surface of an enzyme such as amino groups (NH₂) of arginine or lysine, carboxylic group (COOH) of

glutamic acid or aspartic acid, a hydroxyl group (OH) of threonine or serine, and sulfhydryl group (SH) of cysteine [21]. 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 (Sephacrose). The sugar remains in these polymers comprise ideal functional groups, hydroxyl groups, for covalent bond formation. Also, hydroxyl groups can produce a hydrophilic atmosphere in the acquis solution by forming hydrogen bonds. Bead formed supports are used [22].

In entrapment, enzymes are blocked in the engineered or regular polymeric systems, it is a porous layer that 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 an assortment of cell types, sub-cell organelles, multi-segment frameworks [23]. In ionic bonding, the holding required between the compound and the help material is salt linkages. The idea of this monovalent 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 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 straightforward, 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. This technique of cross-linking for immobilization depends just on the catalyst, is done by joining the compound (or the cells) to one another to set up a huge, three-dimensional complex structure, enzymatic cross-linking typically incorporates the 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 % [24]. Be that as it may, restricting elements can be utilized in this strategy for living cells and numerous catalysts because of destructive materials. To limit the nearby issues that can be found as a result of cross-linking of single enzyme type, both egg whites and gelatin have been utilized [25].

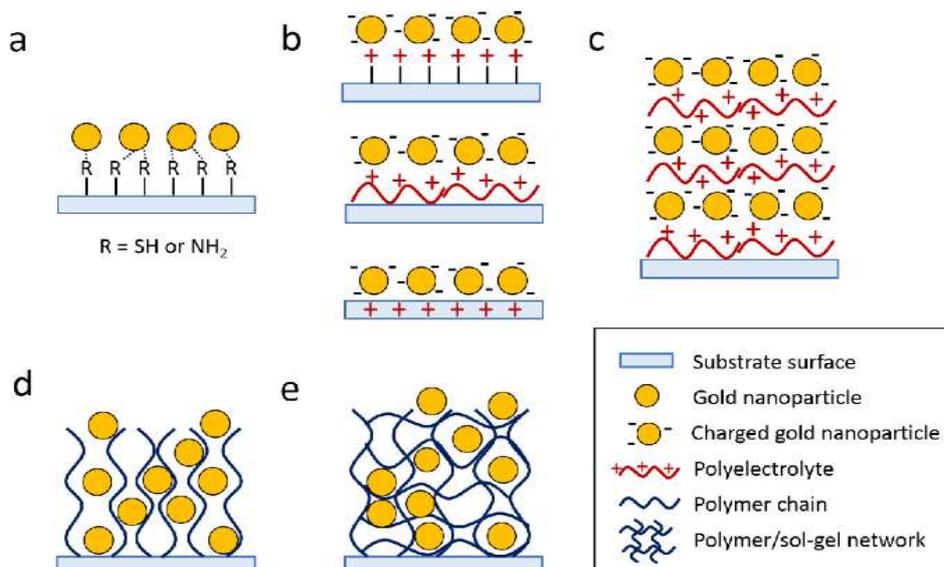


Figure 1. Various surfaces along with nanoparticles used in immobilization.

3. NANOMATERIALS FOR IMMOBILIZATION OF ENZYMES

In recent searches, nanomaterials are now in quick use and are involved in many experiments. There are different types of nanoparticles based on materials used [26].

3.1. Nanometals

By using the metal-organic framework, nanocomposites were formed. MOFs were formed by a metal ions series like Fe^{3+} , Zr^{4+} and La^{3+} , these three ions connected with a material that is 2-aminoterephthalate (H_2ATA) which formed three MOFs. These MOFs then went through an annealing process in a nitrogen atmosphere and this was done at 550°C . From microstructure and morphological analysis, it was revealed that MOFs original structure was retained in this reaction. Then these materials which were derived from the MOF were used for the immobilization [27].

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 [28].

Nanomaterials are being used as matrices for the immobilization of many enzymes like lipase (*Candida rugosa*) enzymes. SWNT and MWNT are mostly used nanoparticles. Firstly, SWNT were used then MWNT was formed, which are used more as nano- biocatalysts [29]. Tin dioxide is used as support for the immobilization of the *C. rugosa* lipase (Nano- SnO_2). 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 45% activity but polypropylene-based immobilization completely inactivated the enzyme. Researchers suggested that the size of the material is needed to be optimized for the maximum loading of the tin dioxide [30].

The material of nanoparticles affects the functioning and immobilization of the specific enzyme. Here is the example of pectate lyase (PL) in which formerly calcium 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 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 [31].

3.2. Gold and silver nanoparticles

Gold and silver nanoparticles are in use for different enzyme immobilization techniques, in these processes either enzyme or the whole cell is used is the example of one enzyme that is alcohol dehydrogenase [32, 33].

Gold nanoparticles were used in the immobilization of the tyrosinase enzyme. Immobilization of enzyme was done by using the solution of gold nanoparticles along with silicate sol-gel matrix and then the assembly was done on the surface of Indium tin oxide ITO electrodes. Aim of using gold nanoparticles was to make the enzyme more stable with better catalytic properties, conductivity, and electron transfer ability, optical and electrochemical properties [34].

3.3. Nanodiamond

Diamonds and graphene are some of the most stable crystalline structures [35]. Nanodiamonds are preferably used in nanobiotechnology as they are biocompatible because of their non-toxic nature and

extraordinary cellular uptake. Nanodiamonds are used in the immobilization of alcohol dehydrogenase which is obtained from the *Saccharomyces cerevisiae*. Nanodiamonds immobilized under optimum pH were observed, which retain 70% activity as compare to the lower efficiency other methods [36].

3.4. Nanofibers

Electrospun nanofibers are recognized as the best support for the immobilization of the enzyme because it gives the best surface area to volume ratio, multiple attachment sites and low limitations of mass transfer. Polyaniline, which is used for immobilization of L-asparaginase, showed the best stability at different pH levels and temperatures. In comparison with some other methods, this material shows stability even after 40 cycles at pH 8.5 and temperature 37°C [37].

Cellulose nanofibers which are mostly used for different methods, are prepared by mechanical or enzymatic methods by using plant fibers. Glucose oxidase is an enzyme immobilized by using a porous matrix, which is of polyaniline nanofibers enzyme. This nanofiber matrix helps in immobilizing the enzyme in three steps: first step is absorption, second is precipitation, third and the last one process is cross-linking [38].

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 component's immobilization. Nanographene oxide (GO) is being used to immobilize different kinds of enzymes. 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 [39].

4. CARBON NANOTUBES FOR IMMOBILIZATION OF ENZYMES

Nanotube chemistry and the method employed for immobilization of enzyme on carbon nanotube influence activity of CNT-enzyme conjugate [40]. Goh and his colleagues merged iron oxide nanoparticles with SWNTs to generate magnetic SWNTs. They immobilized Amyloglucosidase 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 the nanotube from the reaction mixture by magnet detached the enzyme making its reusability possible. Enzyme retained its activity for 1 month at 4°C storage, thus making the enzyme cost-effective for applying on an industrial scale for biofuel production.

In another study, immobilization of lipases and esterase 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 the greater catalytic activity and operational stability as compared to physically adsorbed enzymes.

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 immobilized OPH on SWNT retained higher catalytic activity than OPH immobilized on MWNT [41].

Lipase immobilized covalently on MWNT when subjected to analysis by FTIR spectroscopy and circular dichroism CD, revealed that lipase-MWNT conjugate show less dependence on temperature than free lipase. Further, immobilized lipase had better stability.

Another approach employed controlled placement of enzymes on CNT by using Comb-branched DNA. Foundation DNA strand was covalently attached to MWNT on a glassy carbon electrode. In this approach, comb-branched DNA was prepared using deoxy-ribozyme to bind DNA strand at a peculiar location on the foundation strand. By altering the foundation strand, the placement of DNA strands could be adjusted, which allowed distance optimization between the enzyme and the surface of the 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 [42].

4.2. Non-covalent immobilization

Non-covalent immobilization is a 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 involves π - π interactions and hydrophobic interactions between the enzyme and nanotube surface.

Catalase was adsorbed onto SWNT, oxidized SWNT (O-SWNT) and MWNT. Upon analysis, reduction in catalytic activity was observed mainly in the case of O-SWNT, more in the case of SWNT and less in MWNT. Fourier transform infrared spectroscopy (FTIR) and (CD) revealed a loss in the structure of enzyme which was adsorbed onto MWNT than that on SWNT. An increase in the 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 [43].

Laccase enzyme was immobilized onto MWNT and O-MWNT for investigating the catalytic activity of the immobilized enzyme. The activity was reduced more in the case of MWNT and less in O-MWNT. Structure loss was not observed.

5. APPLICATIONS OF IMMOBILIZED ENZYMES

5.1. Applications of lactase enzyme in the dairy industry

Beta-galactosidase, also known as lactase, is the most important enzyme used in the dairy industry. This enzyme is used to hydrolyze lactose, which is a disaccharide sugar. This is quite beneficial for people who are lactose intolerant. Lactose is the major component of milk products and 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 whey beverages by the addition of lactase [44].

5.2. Applications of pectinolytic enzymes

Pectinolytic enzymes are being used worldwide at an 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 a longer time period and at high temperatures. Moreover, immobilized enzymes can be recovered for reuse [45].

Phototherapeutic properties of pectic substances and their modified products are being studied these days to produce nutraceuticals with application in dietary nutrition and pharmacy [46].

Pectinase pretreatment has the potential for improving the efficiency and environmental friendliness of bagasse soda-anthraquinone 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.

Pectinases are also being used in biorefineries for hydrolyzing pectin present in pectin-rich agro-industrial wastes. Bio-scouring is an eco-friendly method for the removal of non-cellulosic impurities from the fiber with the help of enzymes. Improved results are achieved when pectinase is used to remove sizing agents from cotton safely and eco-friendly, replacing toxic caustic soda.

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 [47].

5.3. Applications of lipases

Lipase enzymes are the most suitable enzymes for catalyzing 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 a wide range of synthesis reactions, like ester bond formation and hydrolysis [48]. Lipases have an 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 used daily in houses to wash clothes and dishes [49].

5.4. Applications of xylanases, amylases and cellulases

These enzymes are used to hydrolyze the plant biomass. Their potential to be used in the energy, fuel and food industries is being studied [50]. 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 [51-53].

6. CONCLUSION

The efficacy and stability of a chemical reaction are increased by using enzymes instead of the conventional methods. The enzyme activity is greatly enhanced by immobilizing them. Conventional methods are not reliable in the sense that they make enzymes less stable and show decreased diffusion. Nanoparticles 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. The non-covalent immobilization method is superior. Industrialists are more concerned about the use of immobilized enzymes because at a larger production scale, enzymes remain active and stable for a longer time period and can be reused. Along with the recent development of immobilized enzymes, we will strive to revolutionize this interesting field.

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