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Review

Bio-Enzyme Hybrid with Nanomaterials: A Potential Cargo as Sustainable Biocatalyst

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Abstract: With advancements in bionanotechnology, the field of nanobiocatalysts has undergone rapid growth and revolutionized various nanomaterials as novel and fascinating nanocarriers for enzyme immobilization. Nanotubes, nanofibers, nanopores, nanoparticles, and nanocomposites have been successfully developed and used as nanocarriers. The construction of robust nanobiocatalysts by combining enzymes and nanocarriers using various enzyme immobilization techniques is gaining incredible attention because of their extraordinary catalytic performance, high stability, and ease of reusability under different physical and chemical conditions. Creating appropriate surface chemistry for nanomaterials promotes their downstream applications. This review discusses enzyme immobilization on nanocarriers and highlights the techniques, properties, preparations, and applications of nanoimmobilized enzymes.

Keywords: enzyme carrier; nanoimmobilized enzyme; nanobiocatalyst; nanomaterial; enzyme assay

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1. Introduction

Enzymes are macromolecular biocatalysts composed of complex globular proteins that play vital roles in all stages of metabolism and biochemical processes in a living system [1]. In addition, enzymes are a significant part in the latest "white biotechnology" trends, including sustainable energy and green chemistry. Enzymes are highly efficient and specific biocatalysts for many reactions, owing to their ability to accelerate chemical reactions by turn-over under mild conditions with high substrate specificity. Enzyme and substrate reactions occur primarily by lock and key or induced fit mechanisms. Biologically active enzymes contain thousands of atoms in specific arrangements that catalyze various biochemical interactions in living biological cells [2]. They have been widely used in various applications in daily life, such as biomedicine, food, biofuel, diagnostics, and bioremediation. Nevertheless, widespread industrial applications and desirable properties are usually limited by a lack of long-term operational stability, recovery, recyclability, and shelf-life. These conditions leave room for further enhancement. Enzyme immobilization is the most efficient method for solving these bottlenecks, resulting in enzyme application in

biotechnological processes. Considering different parameters for enzyme immobilization can accelerate the efficient utilization of enzymes [3].

2. Enzyme Immobilization and Stabilization

The term "enzyme immobilization" is defined as "enzymes physically confined or localized in a certain region of space with retention of their catalytic activities and can be used repeatedly and continuously." Enzyme immobilization has been discovered and used since 1916, as Nelson and Griffin found that "invertase" can absorb charcoal and hydrolyze sucrose [4]. This discovery proved that the enzymatic activity of invertase is not hindered when it is adsorbed on a solid support, such as aluminum hydroxide or charcoal [5]. The dawn of immobilized enzymes has led to the advancement of recently available enzyme immobilization techniques. An ideal enzyme immobilization system has three major components including mode of attachment, enzyme, and carrier. A number of advantages, such as an increase in volume-specific enzyme loading, ease of enzyme reusability in downstream processing, and improved enzyme stability are the main driving forces for enzyme immobilization [6]. Various enzyme immobilization techniques, such as adsorption, crosslinking, and covalent binding, have been developed [7]. Enzymes immobilized in/onto nanomaterials have been established as nanocarriers. Nanostructured materials, such as nanotubes, nanofibers, nanoporous materials, nanoparticles and nanocomposites, are used as enzyme carriers. Nanomaterials have significant advantages owing to their large surface area, which increases catalytic efficiency for commercial applications of nano immobilized enzymes, facilitating reaction kinetics and higher enzyme loading [8]. Hence, the design and application of nanomaterials for enzyme immobilization are essential areas of interest.

The stabilization of immobilized enzymes has been widely studied widely for multiple downstream purposes [9,10]. Proper immobilization enhances enzyme stability via multipoint immobilizations, improves activity, specificity or selectivity, and more activity in the presence of inhibitor or inactivating agents in order to facilitate purifying the enzyme [11,12]. Specificity/selectivity with improved enzyme activity is mandatory for industrial and real-sample analyses because of multiple proteins/interferences in biological samples [13].

3. Enzyme Immobilization Techniques

The choice of the most appropriate immobilization technique is an integral part of the enzyme immobilization process because of its significant impact on enzyme activity and properties in a specific reaction. The ideal enzyme immobilization technique prevents enzyme activity loss by retaining the enzyme active sites' biochemical properties or functional groups. The carrier and the nature of the enzyme strongly govern the selection of the most appropriate technique. Generally, the various approaches for immobilizing enzymes can be classified into two broad classes: chemical and physical. Chemical methods involve the formation of covalent bonds between the enzyme and carrier. In contrast, physical methods involve weaker, noncovalent interactions, namely van der Waals forces, hydrogen bonds, hydrophobic bonds, and ionic bonding. Conventional techniques for enzyme immobilization include covalent binding, adsorption, encapsulation, and crosslinking. Eventually, optimizing enzyme immobilization and the desired the surface chemistry is mandatory for the efficient utilization of enzymes [3,14–17]. Nonetheless, no one method is ideal for immobilizing all enzymes because the abovementioned methods all have advantages and disadvantages (Table 1) and due to the differing types of enzyme immobilization techniques (Figure 1). Furthermore, the use of current cutting-edge techniques and approaches is increasing with advancements in enzyme technologies [18–21].

Table 1. Summary of advantages and disadvantages of various methods of enzyme immobilization.

Method of Enzyme Immobilization	Advantages	Disadvantages
Adsorption	• Simple and easy • Cheap • Chemical-free enzyme binding • No reagents are required • Minimum activation step involved • High retention of enzymatic activity • Reversibility	• Weak physical bonding • Enzyme leakage • Low stability • Desorption of enzyme
Covalent bonding	• Strong biocatalyst bindings • No enzyme leakage • High reusability • High thermostability • High uniformity of the self-assembled monolayer (SAM layer) • Good control of the immobilized enzyme amount	• High risk of enzyme denaturation • Conformation restriction • Limitation of enzyme mobility • Less efficient • Longer time required.
Cross-linking	• Simple • Strong chemical binding • Support-free • Prevention of enzyme leaching • Minimize in desorption. • Possibility for modification of microenvironment	• Harshness of multifunctional reagent used • Loss of enzyme activity • Possibility for enzyme conformational changes
Entrapment/ Encapsulation	• Cheap • Fast • Large surface area • Mild conditions are required. • Improves enzyme stability. • Minimizes enzyme denaturation • Decrease in leaching • Ability to creation of optimal microenvironment	• Limitation in mass transfer • Low loading capacity • Abrasion of support material • Deactivation of enzyme when immobilized

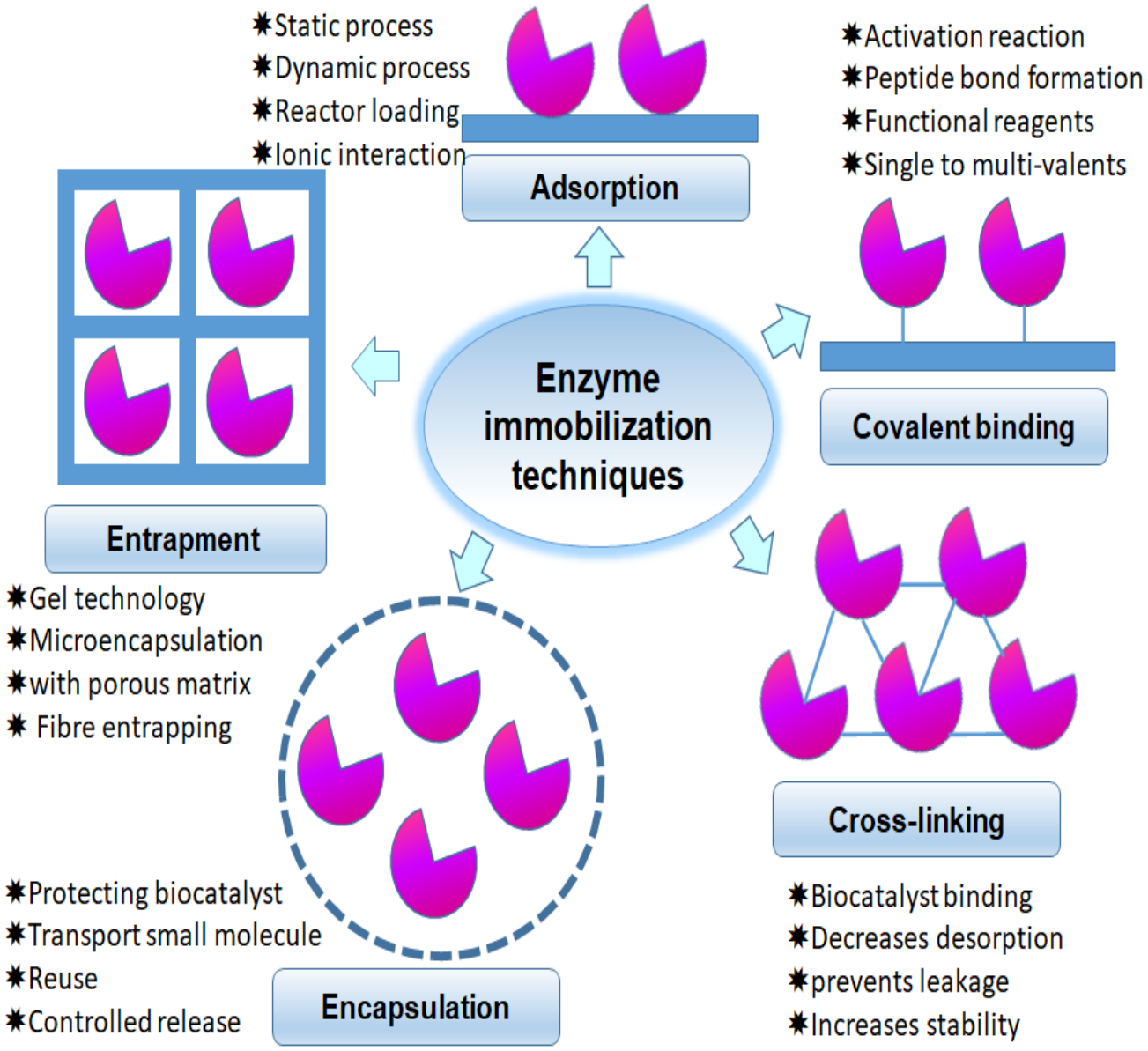

Figure 1. Types of enzyme immobilization techniques. Techniques that work with different principles are shown.

3.1. Adsorption of Enzyme

Adsorption is the oldest and most effortless techniques for enzyme immobilization. This straightforward method for reversible carrier-bound immobilization is widely used and involves electrostatic interactions or passive adsorption. The interaction between the enzyme and the surface of the supporting material is due to weak nonspecific forces, such as van der Waals forces, hydrogen bonds, and hydrophobic bonds. In contrast, enzymes are bound via salt linkages in ionic bonding. Adsorption can be carried out by mixing the enzymes with an appropriate adsorbent under proper temperature, ionic strength, and pH conditions. Enzymes immobilized using adsorption are protected from aggregation, interaction at hydrophobic interfaces, and proteolysis. The enzyme immobilized using adsorption is reversible, allowing detachment from the support material owing to the absence of permanent bond formation.

3.2. Enzyme Attachment by Covalent Binding

Covalent binding is a conventional approach for irreversible enzyme immobilization. This can be accomplished by the formation of a covalent bond between the support material and enzyme. Covalent binding generally occurs between the enzyme surface and functional groups in the support material in the presence of amino acid residues. Covalently bound enzymes can be obtained through various reactive sidechains on enzymes with important critical functional groups, such as sidechains of aspartic acid (carboxyl groups), arginine (amino group), lysine (amino group), glutamic acid (carboxyl groups), serine (hydroxyl group), threonine (hydroxyl group), tyrosine (phenolic group), and histidine (imidazole group). The covalent binding of enzymes depends on various factors, including the binding method, specific conditions when binding, and the carrier material's shape, size, and composition. The covalent binding of enzymes to different materials have been shown to enhance the enzyme half-life and thermal stability. However, this method has disadvantages, such as a high risk of enzyme denaturation and loss of functional conformation of the enzyme when chemical modifications of enzymes to possess a functional group are less efficient because lesser enzymes are immobilized using a high volume of bioreagent and longer time is required.

3.3. Crosslinked Enzyme Aggregates

Crosslinking is also known as carrier-free immobilization because the enzyme can act as a carrier, eliminating the advantages and disadvantages of carriers, and a pure enzymatic system can be obtained. The crosslinking process is conducted with the aid of bi- or multifunctional reagents that act as linkers to link enzyme molecules to three-dimensional crosslinked enzyme aggregates. Surface chemicals are potential agents for creating linkers to form enzyme aggregates [22,23]. Glutaraldehyde is the most common crosslinking reagent because it is easily accessible in large quantities and cost-effective. Using glutaraldehyde as a multifunctional reagent can drastically reduce the modification of enzymes by adding inert proteins such as gelatin and bovine serum albumin during the immobilization process. Another option for crosslinking with an enzyme–receptor is between the amine (on the enzyme) and the carboxyl on the substrate or receptor. The reaction between the amine and carboxyl groups can be activated, then stabilized with 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide- and N-hydroxy succinimide-mediated coupling reactions (Figure 2). Both amine-carboxyl coupling and glutaraldehyde modification of nanomaterials are highly feasible [24]. In addition, thiol (sulfur)—containing amino acids, such as methionine, cysteine, taurine, and homocysteine,—on enzymes can be easily attached to gold materials as substrates. Metal oxides (silicon oxide, titanium dioxide, zinc oxide, etc.) with nanomaterials have the potential to achieve ideal surface functionalization, as oxides are reliable for simple chemical reactions with linkers [24]. The enhancement of oxides or the formation of an oxide surface can be achieved by treating the surface with potassium hydroxide. However, heterofunctional supports have significant advantages, and in a study by Trobo-Maseda et al., heterofunctional amino-epoxy and amino-glyoxyl groups were used. In first step, the enzyme is attached to the support, and in the second step, the intramolecular attachment between the enzyme and the support is enhanced [25]. Heterofunctional support generally elevates enzyme specificity and stability to demonstrate high-performance activity. However, the ratios between the two functional groups must be optimized and the functional groups should not share groups that cause nonspecific attachments or common reactions with different steps [26,27]. At present, and in the past, different materials and polymers have been utilized to produce novel materials that link or encapsulate enzymes [28–32].

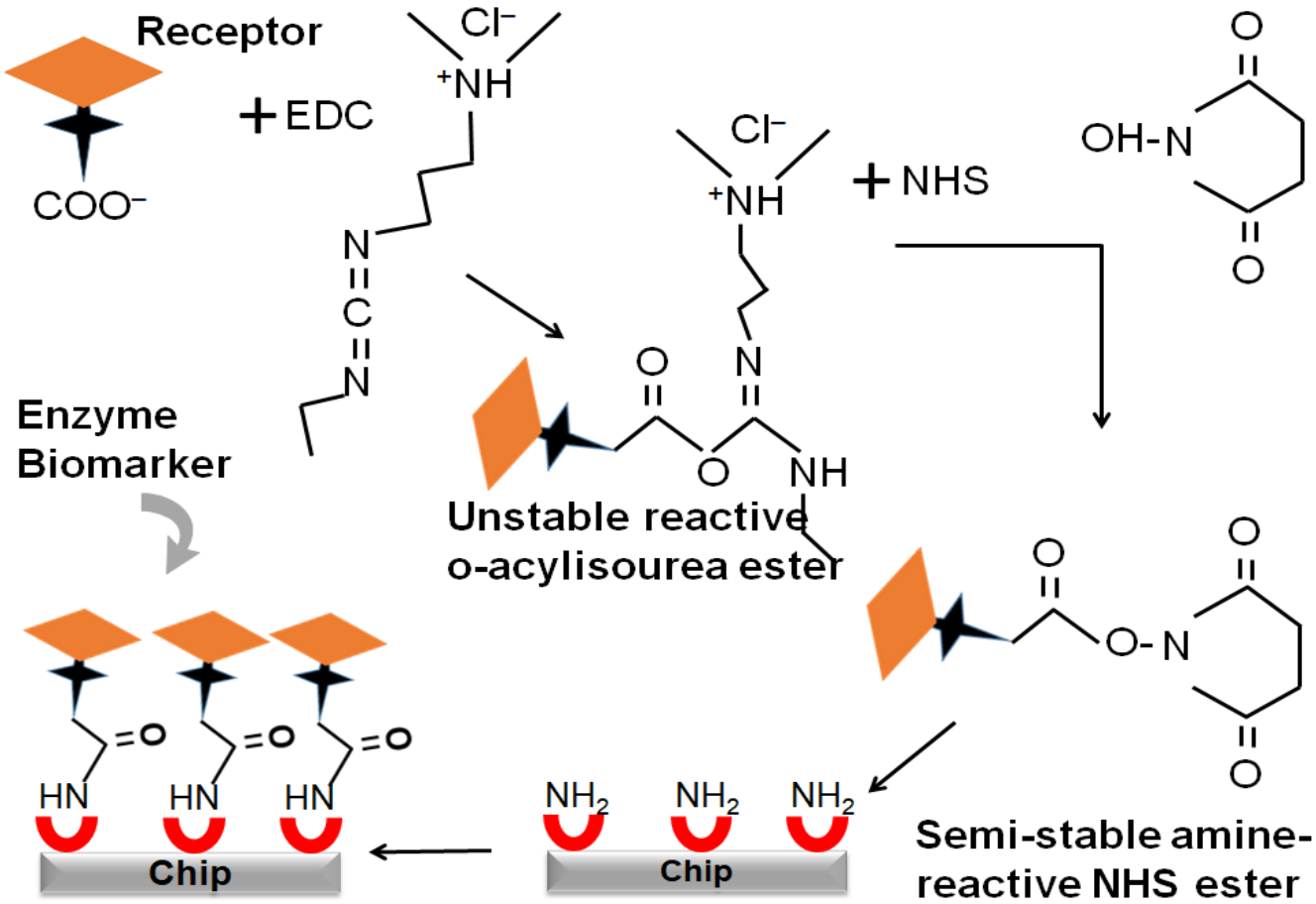

Figure 2. Chemical functionalization strategy. Receptor and enzyme reaction is shown using amine-carboxyl reaction.

3.4. Crosslinking on the Substrate

Nanomaterials for enzyme immobilization utilize functional groups, and the presence of different functional groups on the surface expands their applications. In an investigation, gold nanoparticles and graphene were used to capture the enzyme "glucose oxidase" for sensing purposes. The interdigitated electrode surface material with silica was complexed using the above conjugation, and glucose was detected at a lower level [33]. These two materials (graphene and gold) are currently prevalent in sensing applications, including enzymes. Pure graphene needs to be functionalized; however, activated graphene materials have three easily reacting chemical groups on their surface, namely epoxides, carboxylic acids, and alcohols. Gold is a flexible material that can react with amines on enzymes and proteins and can be tailored to desired sizes [34,35].

3.5. Entrapment of Enzyme

Entrapment is an irreversible approach to enzyme immobilization. Enzymes are entrapped and occluded in the entrapment method within natural or synthetic polymeric networks, a semipermeable membrane that allows only the traversal of substrates and products via diffusion but retains the enzymes [7]. Thus, entrapment is defined as the physical limits of an enzyme within a confined network or space with selectively controlled permeability. The enzyme is not directly bound to the support material and is achieved by covalent or noncovalent bonds when the enzyme is caged within a polymeric network. Entrapment immobilization can be achieved using the inclusion of gels, fiber entrapment, and microencapsulation. This type of entrapment triggers a variety of methods for entrapment immobilization, including photopolymerization, electro polymerization, and microencapsulation.

3.6. Encapsulation of Enzyme

Enzyme immobilization by encapsulation is an entrapment method in which enzymes are enclosed within a membrane. It is a reproducible, simple method that does not require sophisticated equipment [36]. Therefore, encapsulation has attracted significant interest due to its biomolecular freedom and simplicity of preparation compared to other enzyme immobilization methods. During the encapsulation process, enzymes are encapsulated by way of entrapment in a network matrix, such as hydrogels and other polymeric materials, in the form of particles, capsules, and fibers; otherwise, they are encapsulated inside a host-semipermeable membrane [36].

4. Choice of the Carrier for Enzyme Immobilization

The effectiveness and performance of an immobilized enzyme system strongly depends on the characteristics of the carrier. Variations in the physical and morphological characteristics of the support material affects the enzyme immobilization system and its enzymatic characteristics because the carrier is in direct contact with the enzyme. A unique and ideal carrier should have desirable properties, such as chemical and thermal stability, biocompatibility, hydrophilicity, inertness towards enzymes, mechanical resistance, antimicrobial properties, and low cost. Carriers can be classified into two broad categories based on their chemical composition: organic and inorganic. The classification of new materials commonly used as supports is shown in Figure 3. Advances have been made in the use of nanostructured materials called nanocarriers. These nanomaterials fulfill the ideal requirements for enzyme immobilization.

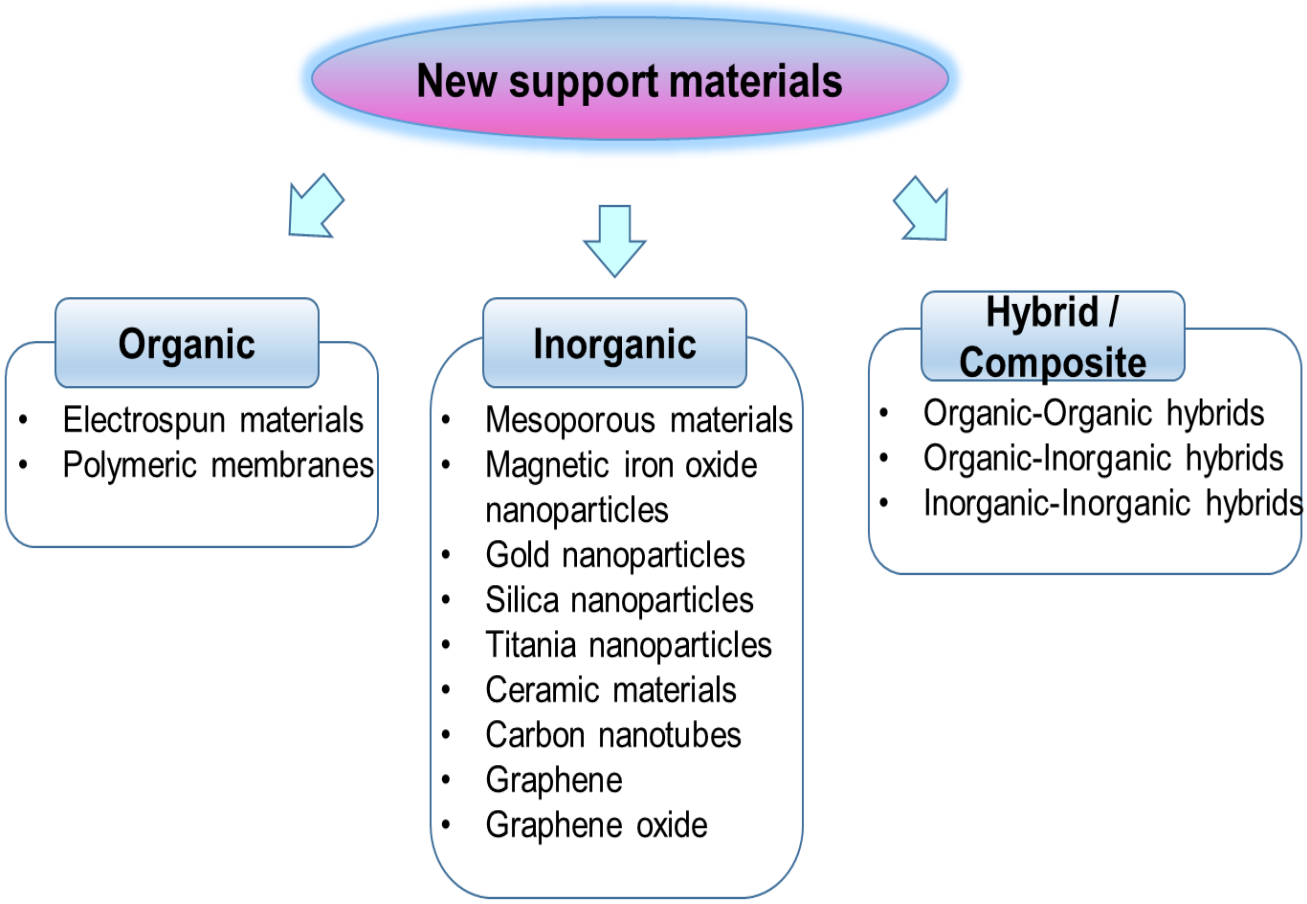

Figure 3. Types of new support materials. All possibilities with organic, inorganic and hybrid materials are displayed.

5. Nanomaterial as Nanocarriers

With the rapid development of nanobiotechnology, using nanostructured materials as nanocarriers in enzyme immobilization system has gained considerable attention. Nanostructured materials are more preferable than conventional materials owing to their unique characteristics, such as hardness, conductivity, nanoscale pore diameter (5–100 nm), hydrophobicity/hydrophilicity ratio, magnetic properties, and defined geometry. These interesting characteristics make them suitable for designing robust biocatalysts. Various nanostructured materials such as nanoparticles, nanofibers, nanopores, nanotubes, and nanocomposites have been successfully developed. The classification of nanomaterials according to their dimensions is shown in Figure 4, namely zero-, one-, two-, and three-dimensional. Nanostructured materials have gained significant attention as carriers for enzyme immobilization owing to their intrinsically large surface areas. Thus, nanomaterials are more preferable than conventional materials because their large surface area allows for increased enzyme loading, resulting in enhanced enzyme activity per volume or unit mass [37]. Biological entities for biocatalysts (enzymes) with nanocarriers can be defined as nanobiocatalysts with unique chemical, electronic, magnetic, and mechanical properties. In addition, when nanomaterials act as nanocarriers, all the benefits of enzyme immobilization on micron-sized particles are successfully inherited.

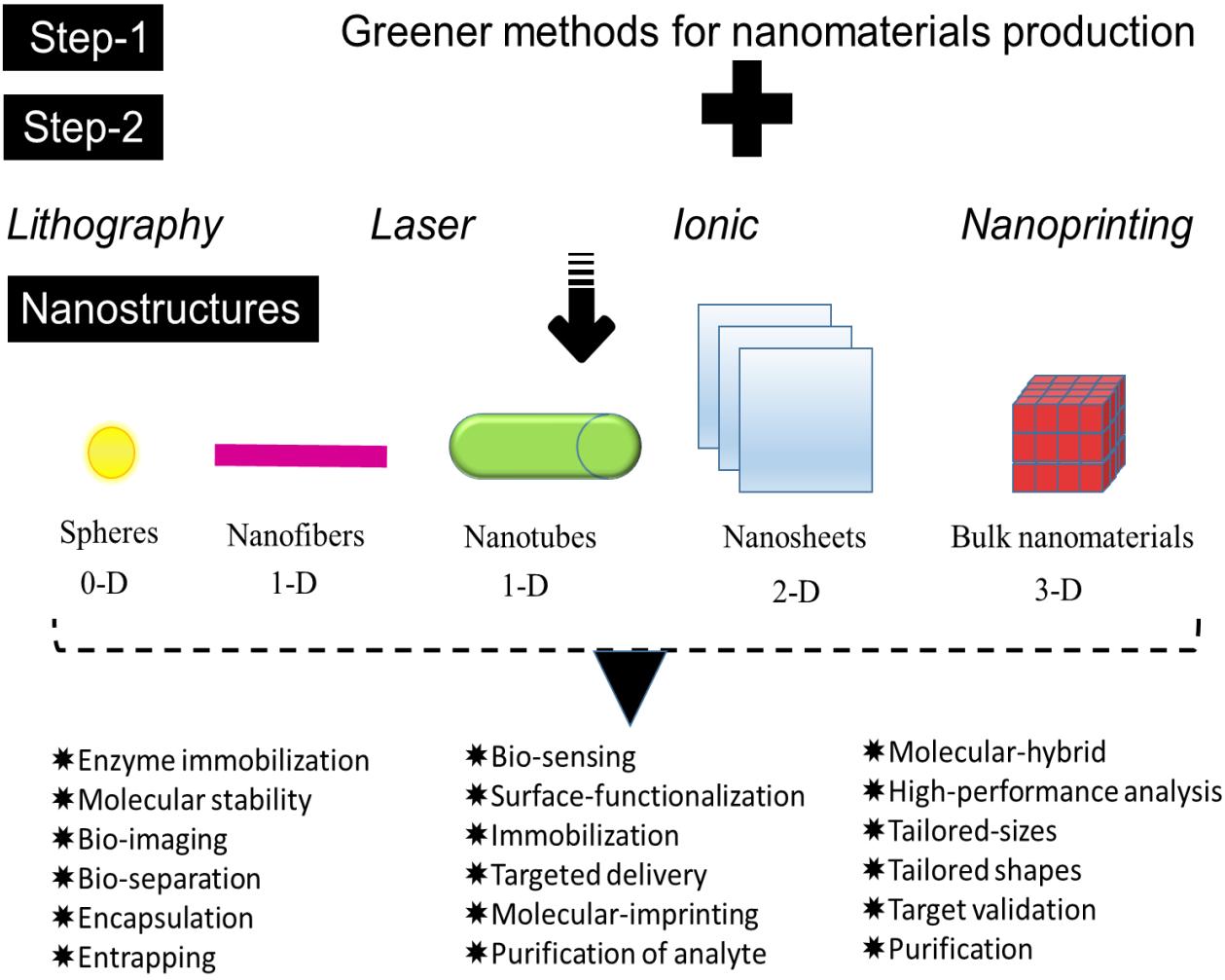

Figure 4. Classification of nanomaterials according to their dimensions. The involvement of other methods such as step-2 along with greener production (step-1) is shown.

Nanosupports must be selected with special attention because several supports are difficult to handle; however, they prevent diffusion problems. It is important to note that if

the support is not porous, the enzyme is exposed to the medium [38]. Nanomaterials have been reviewed as enzyme/biomolecular immobilization platforms in several instances, and their advantages and disadvantages have been discussed [5,12,35,39,40]. For example, graphene and gold are excellent materials that capture the enzyme, providing enhanced performance and stability [33,41].

5.1. Nanoparticles as a Carrier

Nanoparticles are defined as particles (substances) with at least one dimension at the nanoscale (<100 nm). Nanoparticles can exist in agglomerated, aggregated, or fused form and are irregular, spherical, or tubular in shape. Nanoparticles are composed of two parts: a core material and a surface modifier that may be used to change the physicochemical characteristics of the core material. The core materials may be composed of biological materials (chitosan, dextran, lactic acid, lipids, peptides, and phospholipids) or a chemical polymer, carbon, metal, or silica. Nanoparticles, either inorganic or organic, with diameters up to 30 nm, have been broadly studied in recent years as potential carriers for enzyme immobilization.

Recently, nanoparticles have been used as carriers for enzyme immobilization. Nanoparticles play an important role as highly efficient supports for the immobilization of enzymes owing to their fascinating properties in balancing the main elements that regulate enzyme efficiencies, such as enzyme loading efficiency, mass transfer resistance, and specific surface area. A large surface area per unit mass of nanoparticles can increase effective enzyme loading onto nanoparticles by up to 10 wt% [42]. Moreover, nanoparticles are ideal candidates for overcoming relevant diffusion problems when dealing with macromolecular substrates [42]. The enzymatic activity of enzyme-bound nanoparticles is greater than that of unbound enzymes, which was proven by the fact that nanoimmobilized enzymes exhibit Brownian movement during dispersion in aqueous solutions.

5.1.1. Metal-Organic Frameworks

For these applications, metallic and nonmetallic compounds have been formed. Metal–organic frameworks are crystalline, porous, organic–inorganic hybrid materials made of a regular arrangement of positively charged metal ions encircled by organic "linker" molecules. Metal ions act as nodes, connecting the arm of the linkers to create a repeating, cage-like structure. An extensive class of crystalline materials known as metal–organic frameworks have arisen, exhibiting extremely high porosity and large interior surface areas. Different mesoporous and organic materials become key players in conjunction with enzyme capture and encapsulation [20,43–45]. With this set-up, a wide range of downstream applications have become feasible by involving co-immobilization with the surface interface, and structural arrangements have promoted enzyme performance [46–49].

5.1.2. Magnetic Nanoparticles

Generally, magnetic nanoparticles are made up of a highly magnetic core bounded by a polymer shell, whereas the polymer shell is made up of various materials, namely silica, cellulose, and acrylamide, which have properties such as eco-friendliness, biocompatiblilty, nontoxicity, and biodegradablity. The type of polymer shell used for enzyme immobilization is determined based on the application [50]. Nude magnetic nanoparticles do not effectively interact with enzyme particles (proteins); therefore, surface modifications are usually necessary. In this context, compared with other organic nanoparticles, iron oxide nanoparticles have several practical advantages, such as readily forming oxides with ultimate use in surface functionalization. Furthermore, iron oxides are widely used in commercial cosmetic sectors, such as catalysts and pigments, that rely on enzymes. As revealed elsewhere, iron oxide nanoparticles are highly magnetic, unlike other nanomaterials.

Magnetic nanoparticles are suitable as support materials for enzyme immobilization owing to their unique characteristics, such as easy separation under an external magnetic field, large surface-to-volume ratio, superparamagnetism, high reusability, low toxicity,

large surface area, high enzyme capacity, and flexible surface modification using chemical reactions, which significantly improve the loading capacity and reduce the diffusion limitation [51]. In particular, magnetite (Fe_3O_4) nanoparticles and maghemite (γ-Fe_2O_3) nanoparticles are the most prevalent materials that have been used in enzyme immobilization owing to their small size, ease of separation from the reaction media, less toxicity, availability, less environmental impact, good biocompatibility, and superparamagnetic properties [51,52].

Superparamagnetism, which is unique to nanoparticles, is important for its use as an enzyme immobilization support. Superparamagnetic particles, such as Fe_3O_4 nanoparticles, do not have continuous magnetic properties, but they exhibit the phenomenon of "superparamagnetism" when applying an external magnetic field. These particles become magnetized to saturation when an external magnetic field is applied, which makes it easy to purify a high-value product. Superparamagnetic properties are size-dependent and usually arise when the size of the nanoparticles is as small as 10–20 nm. Thus, the synthesized particles must be smaller than 30 nm to achieve superparamagnetic properties; otherwise, ferromagnetic properties replace superparamagnetic properties for particles larger than 30 nm. Immobilizing enzymes onto magnetic nanoparticles provides a simple method for enzyme recovery in reaction media compared to the reusability of enzymes immobilized on nonmagnetic nanoparticles that require high-speed centrifugation [51,53]. Hence, many scientists have studied enzyme immobilization using magnetic nanoparticles because they can be easily separated from reaction solutions using magnetic attraction [53].

5.1.3. Nonmagnetic Nanoparticles as a Carrier

Nonmagnetic nanoparticles made from gold, silver, silica, chitosan, zirconia, and other materials have been broadly used for enzyme immobilization [54]. The nonmagnetic nanoparticle-bound enzyme is fully dispersed in the reaction solution. Thus, regeneration for reuse is often difficult, before long periods of high-speed centrifugation are required.

5.2. Carbon Nanotubes (CNTs) as a Carrier

CNTs are tubular allotropes of carbon composed of graphite. CNTs are a new class of nanomaterials that have attracted significant interest owing to their ideal structure, biocompatibility, and electrical, thermal, and mechanical properties, making them suitable for enzyme immobilization. Among these nanomaterials, carbon nanotubes can act as an interesting support material for enzyme immobilization because of their excellent dispersion in solution and broad factionalization. Carbon nanotubes are classified into two main types: single-walled carbon nanotubes (SWCNTs) (a single graphitic sheet with a tubular structure) and multiple-walled carbon nanotubes (MWCNTs) (an array of nanotubes). A comparison of SWCNTs and MWCNTs is presented in Table 2.

Table 2. Comparison between SWCNTs and MWCNTs.

SWCNTs	MWCNTs
A single sheet of graphene	Multiple sheets of graphene
Catalyst is needed for preparation.	Catalyst is not needed for preparation.
Difficult of bulk synthesis due to appropriate control overgrowth and atmospheric condition is required.	Easy for bulk synthesis.
Easy to twist and are more flexible.	Not easy to twist.
Formation of bundled structures due to not fully dispersed.	No apparent bundled formation with homogeneously dispersed

5.3. Nanofibers as Carriers

Nanofibers are one-dimensional (1D) structures that have attracted significant interest as nanocarriers for constructing nanobiocatalysts because of their extraordinary properties and applications. Carbon nanofibers (CNFs) and polymeric nanofibers are the most com-

monly used nanocarriers for enzyme immobilization. Nanofibers have unique properties, such as homogeneous dispersion in the liquid phase and high enzyme loading, compared to other nanocarriers, and have been studied for nanoeznyme assembly. In addition, the high interconnectivity and porosity of nanofibers offer low mass transfer limitations [55].

CNFs are well-defined cylindrical nanostructures with different arrangements of graphene layers, such as cones, cups, or plates. According to the arrangement of the graphene layers, CNFs can be classified into tubular, herringbone (cup-stacked), and platelet structures. CNFs are preferable to carbon nanotubes owing to affordability, easy mass reproducibility, great surface–active group ratio, and large functional surface area [56]. Polymer nanofibers have remarkable properties, including a large surface-area-to-volume ratio, excellent mechanical performance, and flexible surface functionalities [57]. Several techniques can be used to prepare polymer nanofibers, including electrospinning, chemical synthesis, physical drawing, and nanolithography [58].

5.4. Nanoporous for Enzyme Entrapment

Porous materials are classified into three categories: mesoporous (2–50 nm), microporous (<2 nm), and macroporous (>50 nm), based on the International Union of Pure and Applied Chemistry classification. Materials can be denoted as "nanoporous" if their pore dimension is in the nanometer range (nm). Nanoporous materials comprise carbon, metals, metal oxides, and silica. Nanoporous materials have several fascinating characteristics, such as a high surface area, the ability to immobilize enzymes within the pores and offer a more suitable microenvironment. Mesoporous carbon and silica materials with tunable periodic nanostructures and uniform nanopores have been designed to control the release of small and large molecules [59]. Mesoporous materials have recently gained immense attention as excellent enzyme supports due to their mechanical stability, ease of synthesis, well-defined pore geometry, good pore connectivity, and narrow pore size distribution [41].

5.5. Nanocomposite with Multiple Dimensions

Nanocomposites are commonly classified as fibers (1D), platelets (2D), and particles (3D), depending on the number of nanoscale dimensions. Nanocomposites have recently been developed as enzyme carriers capable of retaining enzyme activity and increasing direct electron transfer between electrodes and redox enzymes [60]. There is a novel trend toward using nanocomposite materials as nanocarriers for enzyme immobilization. Nanocomposite materials have free functional groups specifically positioned on the surface, accessible for specific biomolecule binding in an oriented and controlled manner, or act as a scaffold for simple adsorption. Nanocomposites are commonly fabricated from carbon, sol-gels, polymers and nanometer-sized materials. Nanocomposites can contain catalysts, cofactors, mediators, or stabilizers that may be required for enzyme activity. During fabrication, enzymes can be integrated into two ways: (i) formation of biocomposite paste/ink in a single step together with the other components; and (ii) classical attachment method after preparation of the nanocomposite. The performance of nanocomposites is determined by several nanoparticle characteristics such as aspect ratio, particle size, volume fraction, and biocompatibility with the dispersion and matrix [61]. Nanocomposites can offer better characteristics than their individual components if optimization is achieved.

Enzyme-Inorganic Hybrid Nanoflowers

Different inorganic hybrid nanoflowers have been generated with further nanocomposite applications to capture enzymes, such as cargo [18,62,63]. In the formation of enzyme-inorganic hybrid nanoflowers, different optimization studies in the presence of stabilizing agents such as surfactants and polymers have been conducted [64,65]. Furthermore, these hybrid nanoflowers have various benefits, such as industrial, biosensing, and environmental applications [63,66,67].

Recent advances in enzyme immobilization have focused on developing novel support materials that can enhance enzyme stability, activity, and specificity. Metal–organic

frameworks (MOFs) are highly porous materials with high surface area and tunable characteristics [68]. Enzymes immobilized on MOFs have been demonstrated to exhibit enhanced stability, reusability, and selectivity compared to free enzymes [69]. Furthermore, enzymes immobilized on graphene-based materials have shown unique mechanical, electrical, and thermal properties, making them suitable for enzyme immobilization, because they can enhance enzyme stability and activity by using direct contact and electrostatic interactions [34].

6. Synthesis of Nanoparticle for Enzyme Immobilization

Chemical, physical, biological, and hybrid methods are diverse approaches that can synthesize nanoparticles, as shown in Figure 5. Toxic byproducts that pose environmental risks are formed when nanoparticles are produced using conventional chemical and physical methods. Therefore, nanoparticle synthesis using these methods cannot be used in medicine, especially in the clinical field, owing to health-related problems. Conventional methods have many disadvantages, such as being obsolete, costly, complicated, and inefficient. However, they can produce large amounts of nanoparticles with well-defined shapes and sizes in a short period. Recently, the production of ecofriendly nanoparticles that do not produce toxic waste products during manufacturing has gained increasing attention. With biotechnological methods considered ecologically safe for nanomaterial production, ecofriendly nanoparticles can be obtained by benign synthesis procedures of biological nature as an alternative to conventional chemical and physical methods. The concept of green nanobiotechnology or green technology has developed [70]. Furthermore, analyzing the primary compounds involved in the source material is essential, as they significantly increase the final reduced product.

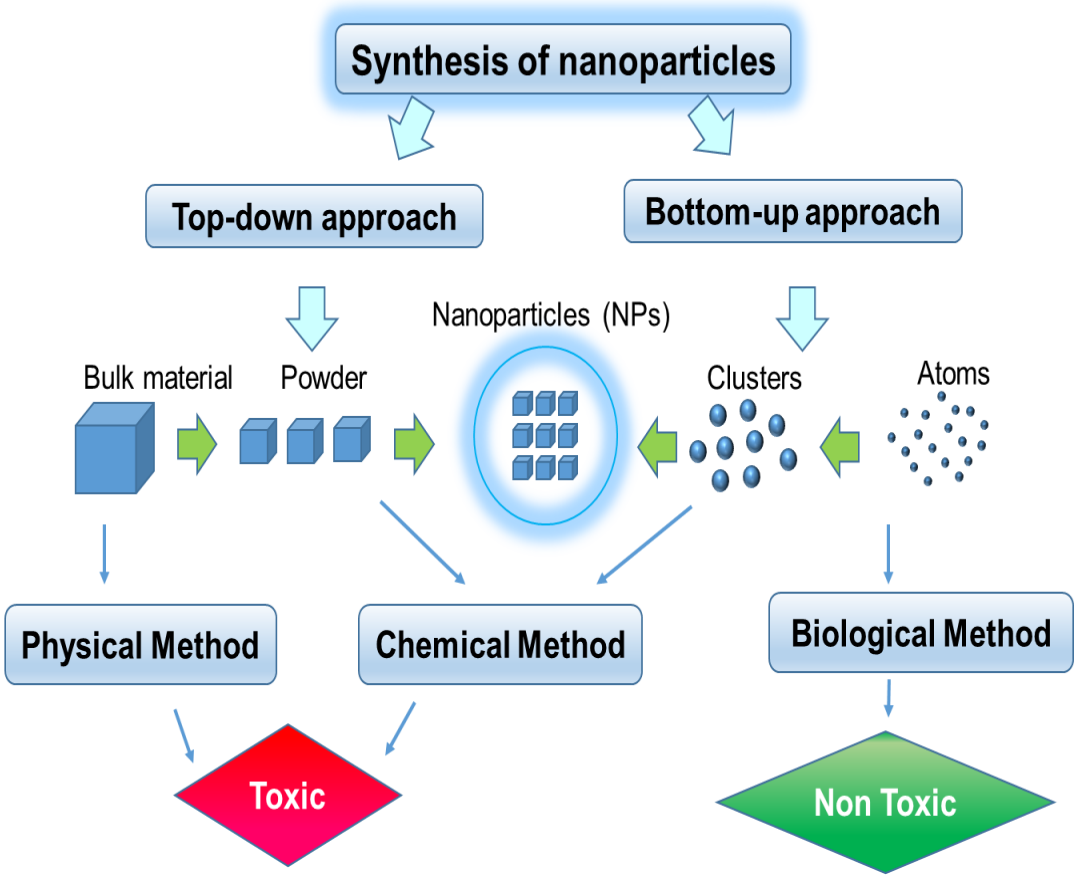

Figure 5. Diagram of nanoparticle synthesis. Single step with greener technology and additional step for the preparation of different nanostructures are shown.

Bionanotechnology uses tools and technologies from nanotechnology to design, synthesize and manipulate biological systems including cells, DNA, and proteins (enzymes). This allows researchers to create new materials and devices that can interact with various biological systems in novel ways. It has been used to develop new drug delivery systems, as well as biosensor, immobilization, and nanoscale imaging technologies [71,72]. Furthermore, it can also be used to create new energy storage and conversion devices, as well as to improve the performance and efficiency of electronic devices [73]. In addition, bionanotechnology has been widely used for enzyme immobilization, which is the process of attaching an enzyme onto the surface of nanomaterials to improve its efficiency, stability, and activity [74].

Green nanobiotechnology involves the production of nanoparticles or nanomaterials using biological pathways with the aid of numerous biotechnological tools. Biological pathways are those related to plants, microorganisms and viruses or the synthesis of lipids and proteins as byproducts [70]. Among these methods, the synthesis of nanoparticles using green technology is far greater than that using chemical and physical methods because green techniques use inexpensive chemicals, produce environmentally benign products and byproducts, and utilize less energy. The biological-based production of nanoparticles using a bottom-up approach can occur with the aid of reducing and stabilizing agents (Figure 5). The three major stages of producing nanoparticles using a biological system are choosing an environmentally friendly reducing agent, solvent medium, and harmless material as a capping agent to stabilize the nanoparticles [70].

7. Conventional Enzyme Assays

Enzyme assays are carried out to serve two different objectives: (i) to detect the presence of a particular enzyme in the sample, a qualitative method in which a clear negative or positive result of the formation of a clear zone is sufficient to determine its presence or absence in a particular specimen (i.e., organism or tissue), and (ii) to determine the amount of the enzyme present in the sample, which is a quantitative method that must deliver data as precisely as possible [75]. Enzymes have a significant advantage, in that they can be determined by their enzymatic reactions, unlike other components of a cell (i.e., nucleic acids or functional proteins), which must be identified using direct detection [75].

The expected results for the qualitative enzyme assays are illustrated in Figure 6a, where the positive results, with formation of a clear zone, indicate the presence of particular enzymes. In contrast, negative results without formation of a clear zone indicate the absence of particular enzymes. The arrow shows the formation of a clear zone. Gopinath et al. [76] revealed that different enzymes could be identified using different types of enzyme assay plates: (i) amylase on starch agar plates, (ii) cellulase on Czapek-dox-cellulose agar plates, (iii) protease on nutrient gelatin agar plates, and (iv) lipase on Tween-20, tributyrin, and LBT agar plates [76]. On the other hand, qualitative assays are a common tool used for screening microbial sources such as fungi and bacteria, providing positive or negative results indicating microbial enzyme production using a microbial. The expected positive and negative results are shown in Figure 6a. Pointing [77] revealed that qualitative assays are very useful method for screening large amounts of fungal isolates for several enzyme classes, because definitive quantitative data are unnecessary. Such tests require only some reagents that are usually inexpensive and readily available. Many factors must be considered for enzyme assays such as pH, temperature, substrate concentration, concentration of the enzyme buffer and ionic strength. These factors cannot be standardized because they differ depending on the types of enzymes testing and the method used.

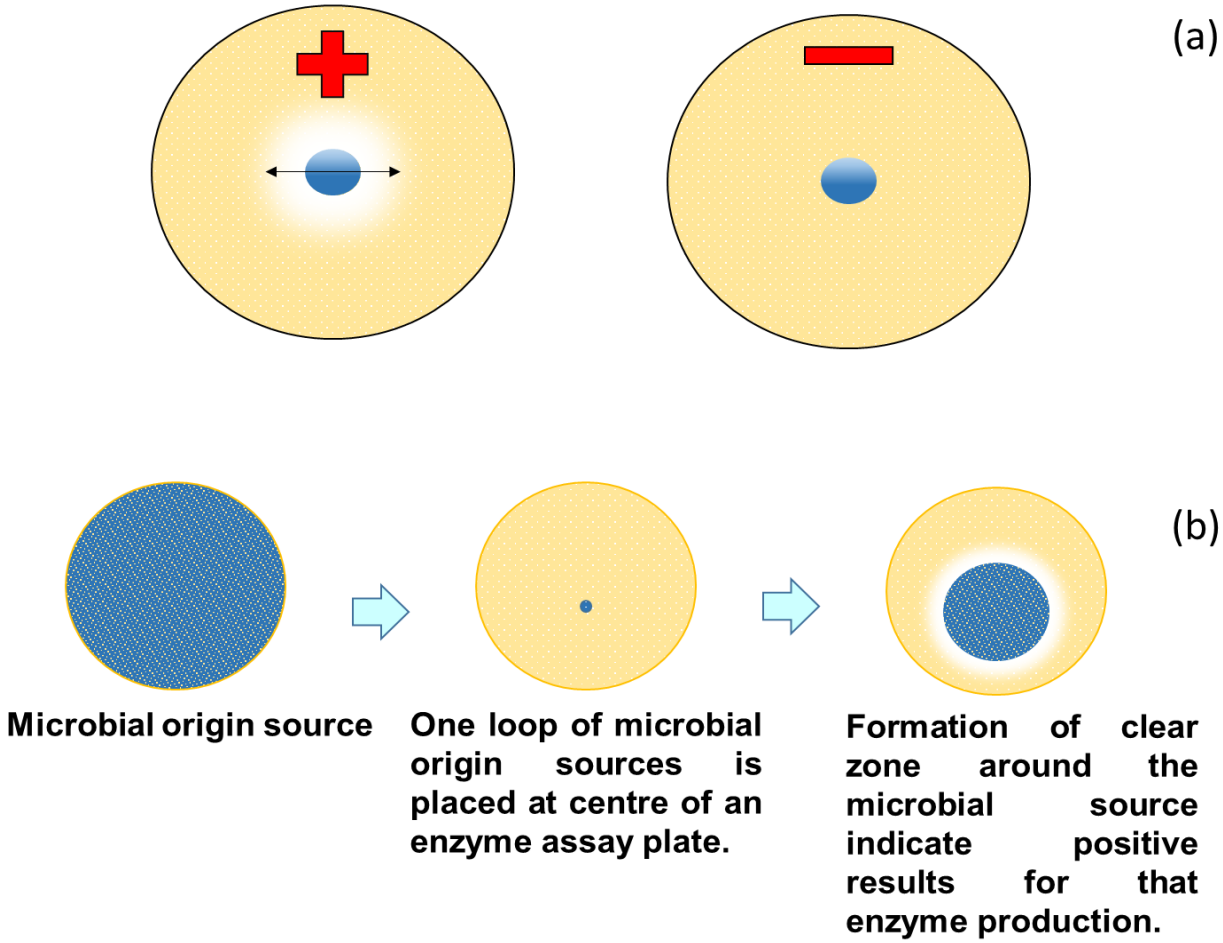

Figure 6. Positive or negative enzyme assay results (**a**). Procedures of pinpoint inoculum assay for screening microbial sources of its ability to produce enzyme (**b**).

7.1. Pinpoint Inoculum Assay on Agar Plate

The pinpoint inoculum method is a qualitative assay used to screen microbial sources such as fungi and bacteria for enzyme production. Previously, enzyme screening studies were performed by Gopinath et al. [78], using different substrates on an agar plate with pinpoint inoculum. Fungi and bacteria were screened for their ability to produce microbial enzymes on solid media such as amylase, cellulase, protease, and lipase. The microbial inoculum was inoculated at the center of the plate, containing the substrates for testing, and then incubated at the optimum temperature for the desired period. The relative activity of microbial enzymes can be estimated by measuring the diameter of the clear zone and microbial growth by the radial limit [76]. The procedure for a pinpoint inoculum assay is shown in Figure 6b.

7.2. Well Diffusion Assay on Agar Plate

The well diffusion assay is the most straightforward method for identifying enzyme activity on solid media. The indicator component is casted onto agar or other suitable gel plates. After that, the supernatant without cells is inoculated into a well in the agar plate [79]. Enzymatic activity can be identified by the zone of clearing or color reaction using a suitable indicator compound. The enzyme concentration is directly proportional to the square of the true zone radius. There is a relationship between the incubation time and clearing area [79]. The procedure for the pinpoint inoculum assay is shown in Figure 7a.

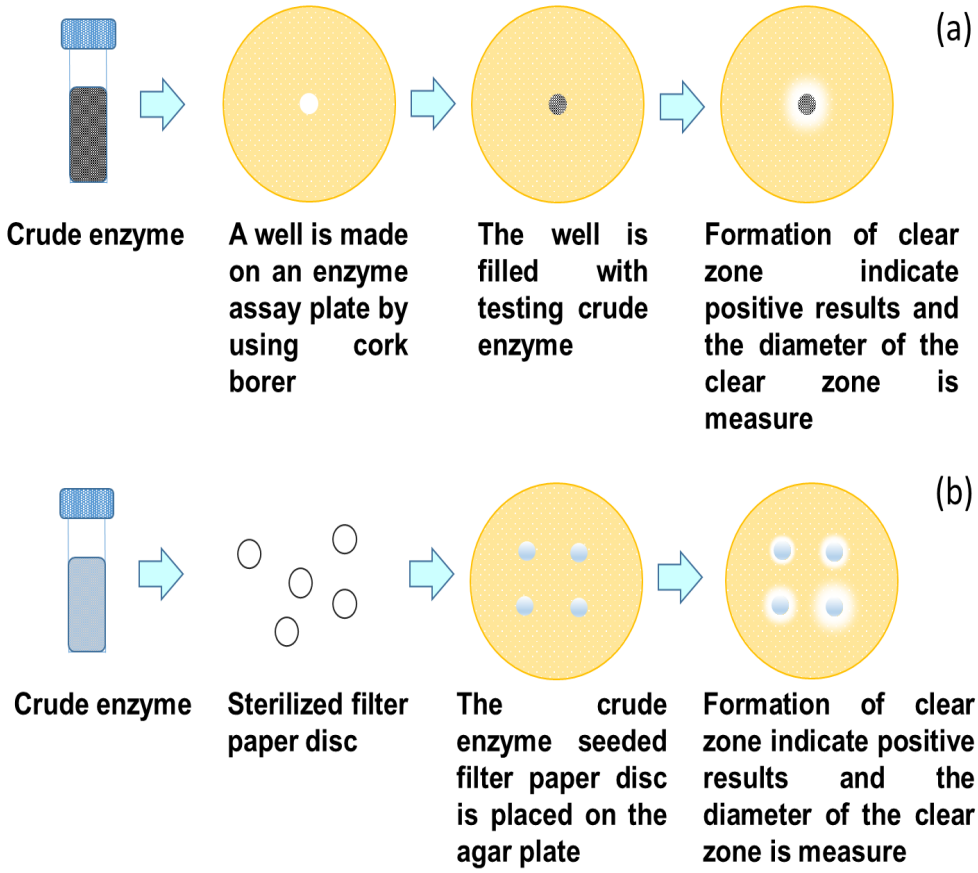

Figure 7. Procedure of well diffusion assay for enzyme activity (**a**). Procedure of disc diffusion assay for enzyme activity (**b**).

7.3. Disc Diffusion Assay on Agar Plate

The principle of the disc diffusion assay is similar to that the well diffusion assay. This is a test of enzyme activity. In this test, filter paper discs containing the enzymes are placed on an agar plate where substrate is placed, and the plate is incubated. If the enzyme reacts with the substrate by diffusion, there is an area around the filter paper disc where the substrate is degraded. This is called the clear zone, and the procedure for the pinpoint inoculum assay is illustrated in Figure 7b.

8. Applications of Immobilized Enzyme to Nanocarriers

Various applications of nanoimmobilized enzymes can be found in the biomedical, biosensor, bioremediation, biofuel, and food industries. Table S1 provides a brief discussion of the potential applications of nanoimmobilized enzymes.

8.1. Biomedical Applications

Therapeutic enzymes are among the most fascinating biomedical applications in the pharmaceutical industry. Collagenase, deoxyribonucleic acid, ribonucleic acid, hyaluronidase, pancreatic, L-asparaginase, L-glutaminase, lipases, urokinase, and streptokinase, are used as therapeutic agents and have great potential in the treatment of cardiovascular, viral, hereditary, oncological, intestinal, and other illnesses [80]. The properties of therapeutic enzymes can be improved using enzyme immobilization on nanocarriers, resulting in enhanced therapeutic power, stability, reusability, life span, and targeting of specific cells (tissues) [81]. The immobilization of therapeutic enzymes on nanomaterials, such as carbon-based nanomaterials, gold nanoparticles, magnetite nanoparticles, maghemite nanoparticles, mesoporous silica nanoparticles, silver nanoparticles, and single-enzyme nanoparticles, has been tested for controlled administration.

8.1.1. Biosensor—A Detection System

Biosensors are analytical devices used for analyte detection. Biosensors consist of three parts: biological sensing components (e.g., enzymes), transducers, and physicochemical detectors. The types of electrochemical transducers used in biosensors include amperometry, conductometry, and potentiometry [82]. Recently, nanoparticles have been used as nanocarriers for enzyme immobilization on biosensor electrodes owing to their high specific area for enzyme binding, high loading capacity and stabilization of enzyme activity by fixing their structural conformation. Enzyme-based biosensors with nanoimmobilized enzymes enable sensitive, fast, timely, and exact detection of compounds. In contrast, traditional detection methods (for example, chromatography) has a slow testing speed, and is often difficult to perform during field operations [83]. Several nanomaterials or nanoparticles, such as carbon, silver, gold nanoparticles, ceria nanospheres, and Fe_3O_4 magnetic nanoparticles have been used for the development of biosensors to detect target molecules [83]. Currently, many biosensor devices associated with numerous enzymes, such as glucose oxidase, horseradish peroxidase, cholesterol oxidase, urease, penicillin acylase, and nanoparticles are widely used in biomedical, clinical, pharmaceutical, environmental, and industrial applications. Considering the enzyme–substrate or enzyme–receptor reactions/interactions, many sensor options are available, including electrical, electrochemical, optical, and mass-based options. Within a sensor, the transducer makes different types of sensing, such as amperometry, voltammetry, conductometry, and potentiometry. However, the biological reaction remains the same for any desired molecule, as observed for the interdigitated electrode sensor, which operates based on the dipole moment among ions on the sensing surface (Figure 8).

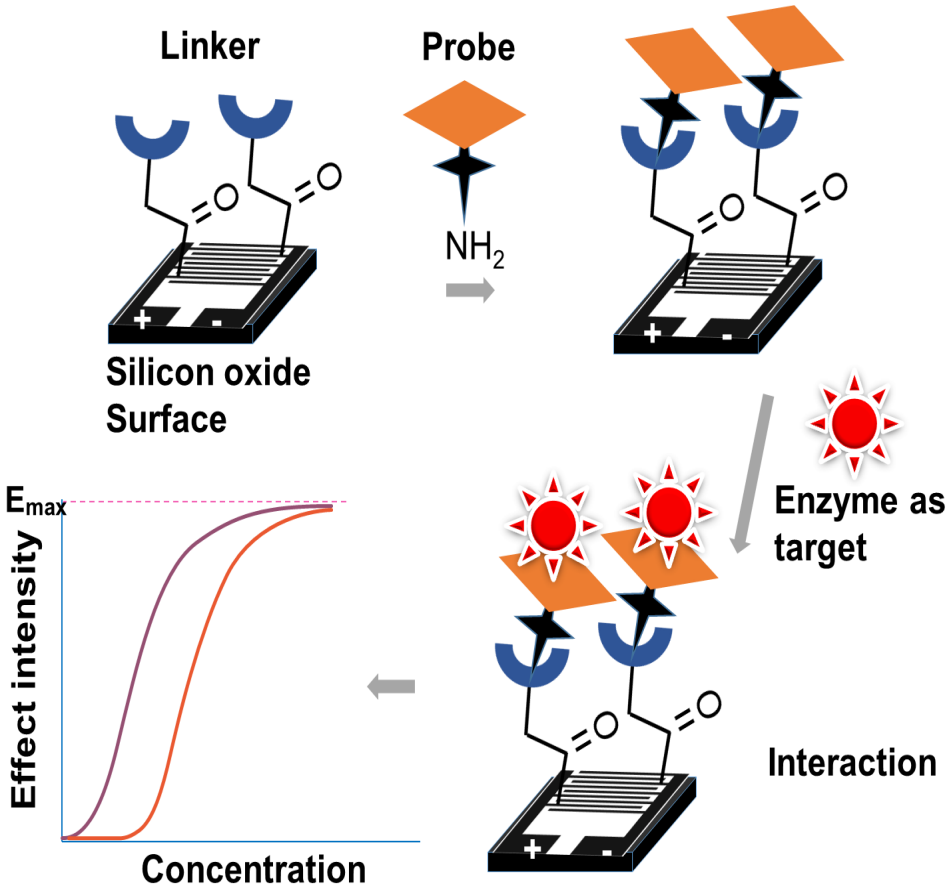

Figure 8. Detection of enzyme on interdigitated electrode sensor. Displayed with probe and target (enzyme) interaction.

8.1.2. Biofuel Production

Biomass is a promising raw material for biofuel production to fulfil current and future demands for sustainable and renewable fuels. Cellulases and lipases are the primary enzymes for widespread applications in enzymatic biodiesel and biofuel production [84]. The rapid growth in biofuel production with enzyme applications has been increasingly observed. Cellulases (EC 3.2.1.4) are used effectively in saccharifying lignocellulosic materials (i.e., plant biomass) for bioethanol production. Cellulase is a combination of three different enzymes, β-glucosidase, endoglucanase, and exoglucanase, working together to yield glucose from lignocellulosic materials and subsequently fermented into bioethanol. Enzyme-based hydrolysis of biomass can be enhanced economically by improving the reusability, thermal stability, and efficiency of enzymes. All of these characteristics can be achieved by immobilizing enzymes on nanomaterials [8]. Nanoimmobilization of cellulases for biofuel production applications has been developed using nanomaterials, such as silica and polymeric nanoparticles [8].

8.1.3. Food Industry

In the food industry, specific characteristics of enzymes, such as storage, reusability, and thermal stability, are essential and should be ensured when employing enzymes under harsh conditions. These characteristics can be achieved by immobilizing enzymes onto nanomaterials. So far, some food-related enzymes, namely α-amylase, β-amylase, diastase, papain, pectinase, lactase, and lipase, have been immobilized on numerous types of nanomaterials comprising nano zinc oxide, graphene oxide–carbon nanotube nanocomposite, gold nanoparticles, silica-coated magnetite (SiO_2-Fe_3O_4) nanoparticles, silica porous nanoparticles, and silica nanospheres.

8.1.4. Bioremediation

Bioremediation of polluted sites using nanoimmoblized enzymes is a new tool for removing environmental contaminants. Enzymatic approaches have gained much attention for decolorizing and degrading azo dyes in wastewater. Enzyme-based bioremediation is a straightforward, easy, fast, environmentally friendly, and nonsocial method for removing recalcitrant xenobiotic compounds from the natural environment [85]. Bioremediation-related enzymes (i.e., horseradish peroxidase, laccase, and lignin peroxidase) have been nanoimmobilized onto various nanomaterials, including chitosan–halloysite nanotube nanocomposites, chitosan-coated magnetite nanoparticles, chitosan nanoparticles, and Fe_3O_4 nanoparticles. The use of nanoimmobilized enzymes in bioremediation has several advantages over traditional approaches. First, it offers a more controlled and sustainable approach to bioremediation. It is possible to create enzymes that are more effective and stable in challenging environmental circumstances, such as high temperatures and low pH levels. Furthermore, the use of nanoimmobilized enzymes enables improved accuracy and selectivity in focusing on certain contaminants, minimizing the impact on nontarget organisms [84]. In addition, enzyme-immobilized nanomaterials have been electrochemically used to detect and destroy water contaminants such as phenolic chemicals, pigments, plastics, medicines, and pesticides [86].

9. Conclusions and Future Perspectives

The creation of novel immobilized enzymes in recent years has resulted in the development of nanomaterials as novel nanocarriers for enzyme immobilization. Recent scientific advances in bionanotechnology have enabled enzyme immobilization on a variety of nanomaterials (nanoparticles, nanofibers, nanoporous, nanotubes, and nanocomposites) with unique and tunable properties. Furthermore, these nanomaterials have desirable properties, such as chemical and thermal stability, biocompatibility, hydrophilicity, inertness towards enzymes, mechanical resistance, antimicrobial capabilities, and low cost, making them the ideal carriers for enzyme immobilization. The enzymatic activity of enzyme-bound nanomaterials is greater than that of unbound enzymes, which is proven

by the fact that nanoimmobilized enzymes exhibit Brownian movement when dispersed in aqueous solutions. Nonetheless, an ideal enzyme immobilization technique requires the construction of a nanoimmobilized enzyme with no loss of enzyme activity after immobilization. Enzyme immobilization in/on nanocarriers can be further developed to enlarge the catalytic repertoire of nanoimmobilized enzymes for widespread application in various fields, such as biomedicine, biosensors, biofuels, bioremediation, and the food industry.

In general, direct precursor reduction under ambient conditions is a standard method for generating nanomaterials that can be undertaken in low-resource laboratories. The capping of compounds from plants seems promising and can be scaled up easily. For scaling-up purposes, the optimum conditions, such as temperature, reaction time, and usage of the right precursor, are mandatory. Various nanostructures have been created in several downstream enzyme-based applications. In general, conventionally generated nanoparticles are spherical in shape; however, their size might vary depending on the intended condition(s). However, owing to several practical requirements, different nanostructures are required to enhance surface areas. Surface expansion by modifying the nanostructure has been implemented in biomolecular capture and nanosensors for enzyme development. The crucial part of generating different nanostructures requires the development of additional methods. The popular techniques include lithography, nanoprinting, exposure by laser, and ionic implantation as bottom-up approaches.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su15097511/s1, Table S1: Potential applications of nanoimmobilized enzymes. References [87–109] are cited in the supplementary materials.

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