



Review

# Recent Advances in Enzyme Immobilisation Strategies: An Overview of Techniques and Composite Carriers

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**Abstract:** For over a century, enzyme immobilisation has been proven to be a superior strategy to improve catalytic activity and reusability and ensure easy separation, easy operation, and reduced cost. Enzyme immobilisation allows for an easier separation of the enzyme from the reaction mixture, thus simplifying downstream processing. This technology protects the enzyme from degradation or inactivation by harsh reaction conditions, making it more robust and suitable to be used in various applications. Recent strategies of immobilisation methods, such as adsorption, cross-linking, entrapment or encapsulation, and covalent bonding, were critically reviewed. These strategies have shown promising results in improving enzyme stability, activity, and reusability in various applications. A recent development in enzyme immobilisation in nanomaterials and agrowaste renewable carriers is underlined in the current review. Furthermore, the use of nanomaterials and agrowaste carriers in enzyme immobilisation has gained significant attention due to their unique properties, such as high surface area, high mass transfer, biocompatibility, and sustainability. These materials offer promising outcomes for developing more efficient and sustainable immobilised enzymes. This state-of-the-art strategy allows for better control over enzyme reactions and enhances their reusability, leading to more cost-effective and environmentally friendly processes. The use of renewable materials also helps to reduce waste generation and promote the utilisation of renewable resources, further contributing to the development of a circular economy.

**Keywords:** enzyme immobilisation; stability; catalytic activity; nanocarrier; renewable carrier; agrowaste



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## 1. Introduction

The effective use of soluble enzymes as green catalysts may be hampered by their drawbacks, such as their non-reusability, high sensitivity to several denaturing agents, high cost, non-stable for large-scale processing, conformation change, not able to reuse and non-applicable in fixed-bed reactors [1,2]. Over a hundred years, numerous immobilisation strategies have been developed to enhance the catalytic activity, stability, storage stability, and reusability and reduce the downstream processing cost. The extensive innovative

approaches of enzyme immobilisation have been investigated. Many of these undesirable constraints may be resolved using enzyme immobilisation [3,4]. Until now, enzyme immobilisation has been a remarkable strategy for large-scale applications due to the ease in catalyst recycling, reusability, continuous operation, easy enzyme separation, wide choice of reactors, easy product purification, and lower cost of downstream processing [5,6]. This outstanding approach has proven to be more stable for catalysis than the use of a soluble counterpart. The concepts of reusability, stabilisation, and lower downstream processing cost have been persistent key factors for the application of immobilised enzymes, thus remaining popular in industrial applications.

Numerous strategies of enzyme immobilisation have been investigated by researchers ranging from methods of affinity adsorption [7], covalent binding [8], cross-linking [9,10], entrapment/encapsulation [11] in reverse micelles and emulsions [12]; organic polymers such as polyallylamine [13], activated carbon [14,15] and chitosan [16]; and inorganic polymers such as nanographene oxide [17], nanosilica [18,19], iron oxide [20], and nanogold [21]. Regardless of the techniques used, enzyme immobilisation still faces obstacles such as lower catalytic performance compared to soluble enzymes, substrate inhibition, mass transfer restrictions, expensive, enzyme leaching, carrier compatibility, and scale-up challenges. Thus, more studies on enzyme immobilisation are required to synthesise an immobilised enzyme that is stable, reusable, high catalytic activity, cost-effective, and suitable for large-scale manufacturing.

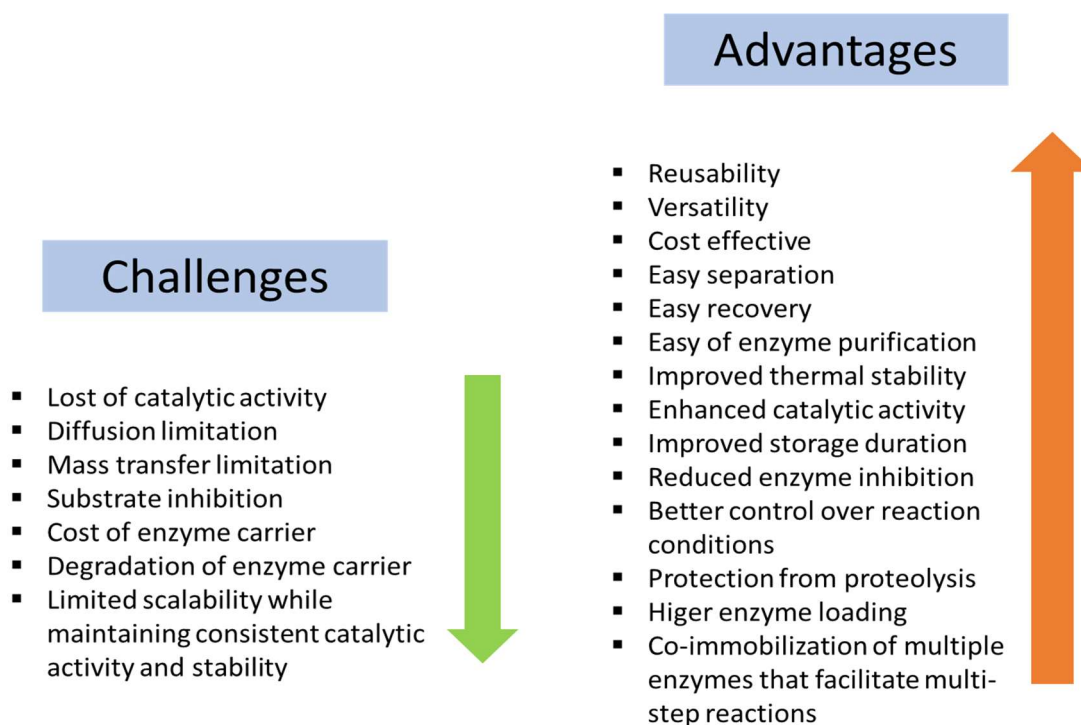
Currently, enzyme immobilisation using nanocarriers has emerged as a promising solution for enzyme immobilisation due to its unique properties and versatility. These nanoscale carriers can efficiently encapsulate enzymes, protecting them from harsh environments and enhancing their stability. Additionally, nanocarriers offer controlled release capabilities, allowing for precise dosage and prolonged enzymatic activity [22]. Additionally, agrowaste materials have recently gained attention as potential carriers for enzyme immobilisation. These materials, derived from agricultural by-products, are abundant, renewable, and cost-effective. They provide a sustainable alternative to traditional carriers and can effectively immobilise enzymes, allowing for their reuse and reducing waste generation. Furthermore, agrowaste materials offer biocompatibility and can enhance enzyme stability and activity in various applications [23]. This review discusses recent enzyme immobilisation methods and their carriers, emphasising state-of-the-art nanosized and agrowaste carriers. This review contributes to illustrating how enzyme immobilisation may improve enzyme stability, reusability, and catalytic activity while lowering costs and simplifying the operational process. Adsorption, cross-linking, entrapment/encapsulation, and covalent bonding are among the methods discussed in this review. The present review emphasises the utilisation of state-of-the-art nanomaterials and renewable agro-waste carriers, which have advantages such as large surface area, biocompatibility, and sustainability. These developments in enzyme immobilisation make it easier to control enzyme reactions and lead to more cost-effective and environmentally friendly operations. This review spotlights the importance of selecting the best immobilisation technique for the enzyme, carriers, substrate, and application, as well as offering insights into the recent advancements and challenges in enzyme immobilisation.

## 2. Overview of Enzyme Immobilisation Techniques

Enzyme immobilisation is defined as the restriction of enzyme mobility in a fixed space in order to increase the catalytic activity, stability, and reusability of soluble enzymes [24–28]. They are physically confined or localised to a certain defined region of space with retention or to enhance their catalytic activities, can be used repeatedly, and are easily recovered [28]. Immobilised enzymes are an insoluble form [29]. It is essentially a specialised form of heterogeneous biocatalysis. As a consequence of enzyme immobilisation, some properties, such as catalytic activity, thermal stability, and storage stability, became altered [30,31].

Among the benefits of enzyme immobilisation mentioned above, the primary disadvantages of using immobilised enzymes include a decrease in enzyme activity during the

immobilisation process, particularly when the enzymes are coupled to macromolecular substrates [12]. Diffusion limitations, enzyme leaching, high cost, and scalability are other major drawbacks related to this technology [32]. Therefore, continuous effort is required to enhance the catalytic activity, stability, reusability, and easy recovery of immobilised enzymes so that they can be applicable in a wide sector such as catalysis, adsorption, pharmaceuticals, food processing, and biofuel production. Figure 1 depicts the benefits and drawbacks of enzyme immobilisation.



**Figure 1.** Advantages and challenges of enzyme immobilization.

Three different methods can be used to immobilise enzymes on a support material: adsorption, cross-linking or covalent bonding, entrapment, and/or encapsulation [33]. The main elements of an immobilised enzyme system are the enzyme, the carrier, and the mode of attachment [34]. The process of immobilisation significantly impacts the biocatalyst's characteristics; hence, the immobilisation strategy chosen will ultimately influence the biocatalyst's stability and catalytic activity [35].

### 2.1. Adsorption

Adsorption can be defined as the process by which an enzyme accumulates on a solid surface support due to intermolecular interactions [36,37]. There is no need for surface modification or linkers such as glutaraldehyde and cysteine in cross-linking and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide in covalent bonding [38,39]. Surface adsorption interaction with enzymes involves hydrogen bonds and electrostatic interactions [40]. The interaction of enzymes with support surfaces is vital to the adsorption process. Thus, the polarity and charge of the enzyme are crucial characteristics for ensuring high and repeatable enzyme coverage on the support. This produces generally mild, simple, economic, fast, good performance, and reusability [41]. However, enzyme immobilisation via adsorption encounters disadvantages such as enzyme leaching and enzyme binding to the support often weaker compared to covalent bonding [42].

### 2.2. Cross-Linking

Cross-linking is the chemical process of uniting two or more molecules via a covalent bonding [43–45]. Cross-linked enzyme aggregates are a versatile approach for enzyme

immobilisation. The approach is frequently preceded by a surface modification or activation process [46–48]. Silanisation, or coating the surface with organic functional groups using an organofunctional silane reagent, is a frequently used technique for the initial surface modification of inorganic supports. Using p-nitrobenzoyl chloride, such as coating or natural surface amino groups, can be derivatized to arylamine groups or aldehyde groups [49]. The procedure is easy, comprising precipitation from an aqueous buffer, followed by the cross-linking of the resultant physical aggregates of enzyme molecules, which can be quickly optimised [50]. The resultant immobilised enzyme via cross-linking is stable and functional as a biocatalyst. The disadvantages, however, include often limited activity retention, poor reusability, low mechanical stability, and challenges in handling the gel-like cross-linked enzymes [51].

### 2.3. Entrapment or Encapsulation

Enzyme immobilisation via entrapment refers to the caging of enzymes with covalent or non-covalent bonds. These matrices minimise conformation change and retain the properties of the biocatalyst [52–54]. The enzyme captured inside a network of polymers permits the passage of substrates and products but retains the enzyme [55]. Enzymes are not tied to the polymeric matrix after entrapment, but their diffusion is restricted. An entrapped enzyme is more stable than a physically adsorbed enzyme. Entrapment immobilisation is less difficult to produce than covalent bonding, although enzyme activity is increased or preserved [56–58].

The immobilisation of enzymes via physical entrapment has a broad range of applications and may have less interference with the natural enzyme's properties [59]. However, the encapsulation strategies must take into account the chemical conditions of the polymerisation matrices, the pore diameter and volume, and the compatibility of enzymes with the pore matrices, to ensure that the substrate and product can diffuse in and out of the polymer matrices. Catalytic activity retention is fairly prevalent since many enzyme encapsulations do not result in a significant loss in enzyme activity after immobilisation [60].

Encapsulation is similar to entrapment because the enzyme is confined in a polymer matrix, but the difference is that the polymer support matrix has “pockets” or “pores” to immobilise enzymes [61]. Encapsulated enzymes improve enzymatic performance by modifying hydrophobic interactions, improving reaction surface area, and improving intermediate concentration. They are more stable under a variety of circumstances. They are widely utilised in sectors such as biocatalysis, biosensing, enzyme treatment, biomedicine, and bioremediation [62].

A wide range of enzymes has been encapsulated or entrapped in sol–gel glasses. They maintain their catalytic activity and are accessible to external substances, but diffusion occurs within the silica matrices. Sol–gel glasses are formed into particular forms and are optically transparent, allowing optics and catalytic activity to be combined to create biosensors. The high specificity and sensitivity of enzymes make it possible to detect chemical components. Caresani et al. [63] investigated the encapsulation of *Bacillus subtilis*, *Aspergillus oryzae*, and barley  $\alpha$ -amylases in a silica-based matrix using an acid-catalysed sol–gel process. Biocatalytic activity has been compared between immobilised and free systems, with high surface area xerogels and granular morphological structures.

Enzymes enhanced their bioactivity in the sol–gel silica matrices because of the diffusion or mass transfer through the pores. Entrapped or encapsulated enzymes can be protected from the denaturation effect via the porous sol–gel matrices. The restricted diffusion of external chemicals via the pores of the matrix may considerably diminish environmental impacts that would otherwise degrade the catalytic activity of enzymes. Free enzymes might lose their catalytic activity, but enzymes that have been immobilised might be stable for weeks or even months.

#### 2.4. Covalent Bonding

The establishment of covalent bonds within each molecule of an immobilised enzyme and the support results in a region of the enzyme in which the residues attached to the support remain in their position during any dislocation or movement of the enzyme molecule that occurs due to harmful conditions such as heat, extremely low or high pH, and organic solvents [64]. If the structure of the support is sufficiently compatible with the surface of the enzyme (e.g., a flat support structure and a moderately flexible globular protein), the highly rigidised region can be relatively large, establishing the vital stabilisation of the enzyme molecule as protection against any harm condition such as extremely high acidic or alkaline conditions, and temperature [65].

Parmegiani Marcucci et al. [66] investigated the immobilisation of *Burkholderia cepacia* lipase using the SBA-15 by covalent bonding, which was made with different pore sizes (S8 with 9.1 nm and S23 with 23 nm) and modified with tin (SnS8 and SnS23). The support with the greatest pore size (25 nm) resulted in the highest specific activity of the immobilised enzyme. No lipase was found in the buffer solution used for lipase desorption during the leaching experiments, thus demonstrating a strong bonding between lipases and modified silicas. Zhang et al. [67] disclose the effective synthesis of PNGase F with a glutamine tag in *Escherichia coli* and the site-specific covalent immobilisation of PNGase F with this peculiar tag via microbial transglutaminase (MTG). PNGase F was immobilised from the glutamine tag being covalently and site-specifically converted to primary amine-containing magnetic particles with the use of MTG. With comparable enzymatic efficiency to that of its soluble counterpart, immobilised PNGase F may deglycosylate substrates and demonstrate strong thermal and reusability capacity. Tvorynska et al. [68] examined four covalent methods for laccase attachment:  $\text{NH}_2$  can be supported in the following ways: (i) via glutaraldehyde; (ii) via disuccinimidyl suberate; (iii) using EDC/NHS for Lac coupling with its COOH groups to support  $\text{NH}_2$ ; and (iv) using EDC/NHS to support COOH. Additionally, five supports (cellulose, carbon-based (glassy carbon, graphite) powders, mesoporous silica (SBA-15, MCM-41) were studied. Various immobilisation techniques and supports had a considerable impact on the quantity of the immobilised laccase and, consequently, the analytical properties of the resulting biosensors. Table 1 shows the summary of the methods and materials used in enzyme immobilisation, as well as its benefits and drawbacks.

**Table 1.** Summary of the methods and materials used in enzyme immobilisation together with its advantages and disadvantages.

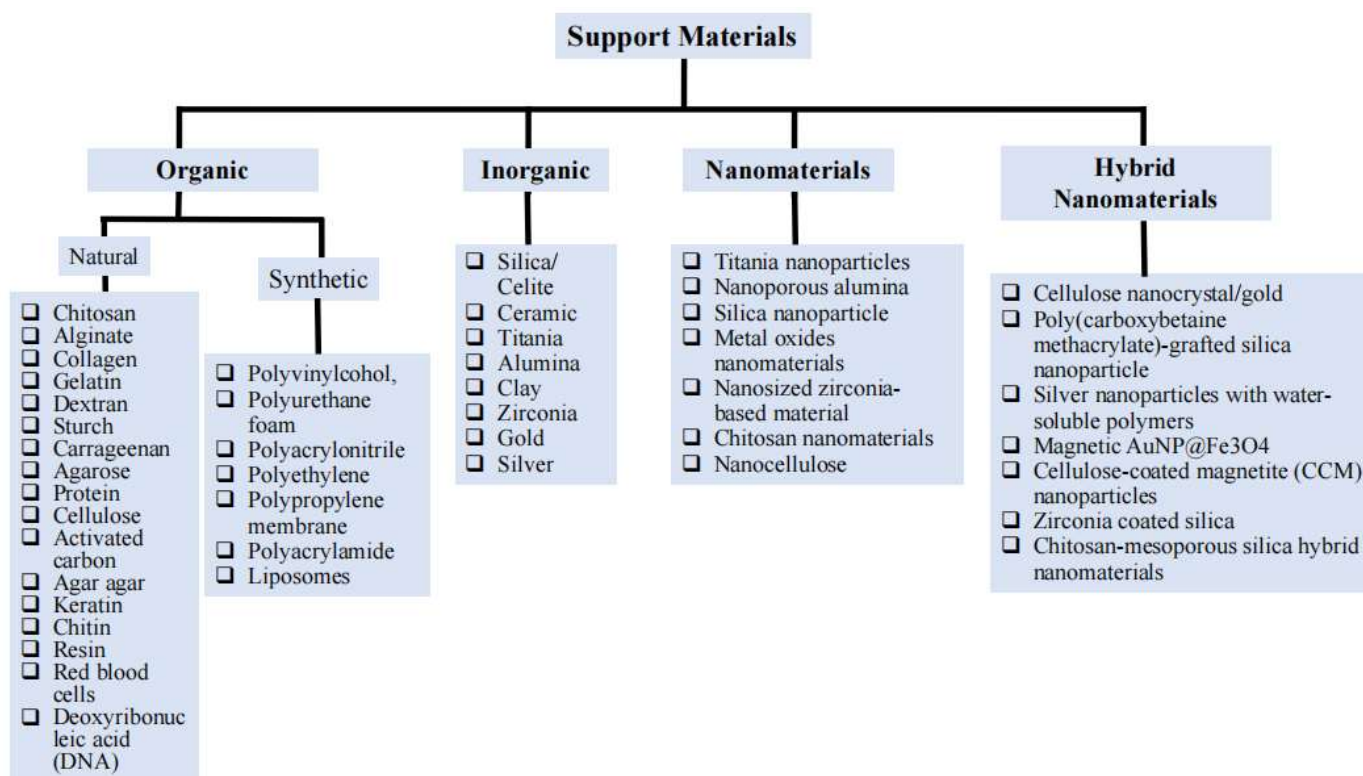
Method	Materials	Advantages	Disadvantages	References
Synthesising magnetic-cross-linked enzyme aggregates (CLEAs) or trapping the CLEAs in particles with superior mechanical qualities.	Glutaraldehyde as a linker; Ammonium sulfate as a precipitating agent; Bovine serum albumin (BSA) and 3-aminopropyltriethoxysilane (APTES) as additive.	It is suggested to tackle substrate diffusion issues by generating more porous CLEAs, among other things.	Diffusion limitation.	[33]
Immobilisation of protease via covalent bonding on chitosan.	Chitosan as a carrier; glutaraldehyde and ethylenediamine as modification agents and cross-linkers.	The catalytic activity of immobilised enzymes is equivalent to that of free enzyme (pH 9 and 60 °C); however, the immobilisation procedure broadened the optimum temperature range of enzyme activity (50–70 °C).	After three cycles of use, the immobilised enzyme only maintained 47.08% of its initial activity.	[36]

Table 1. Cont.

Method	Materials	Advantages	Disadvantages	References
Laccase was immobilised on a low-cost, nanosized magnetic biochar (L-MBC) via adsorption, precipitation, and cross-linking.	Bagasse biochar as a carrier; ammonium sulfate as a precipitation agent; glutaraldehyde as a cross-linker.	The magnetic biochar could immobilise a substantial quantity of enzymes with increased catalytic activity (2.251 U per mg MBC), better stability, improved storage stability, pH tolerance, and thermal stability over free laccase.	The catalytic activity of immobilised laccase decreased more than 50% of its initial activity at pH 5.5 and 6.	[69]
Encapsulation of laccase using alginate, alginate–silica and silica sol–gel.	Tetraethyl orthosilicate (TEOS) and alginate as carriers.	The experimental results showed that incorporating silica into alginate resulted in a better (70%) encapsulation efficiency (EE) for the laccase extract than for the alginate alone (59%). Furthermore, encapsulating the laccase extract in sol–gel resulted in an increase in its catalytic activity, as well as a 90% rise in the EE. The alginate and sol–gel matrices also improved laccase catalytic efficiency compared to free laccase, with $k_{cat}$ values of 89.9 (alginate), 63.7 (alginate-silica), and $56.9 \text{ min}^{-1}$ (silica sol-gel), respectively.	After three reaction cycles, the catalytic activity of immobilised laccase with alginate-silica was decreased by 50%.	[61]
New enzyme immobilisation ideas via multipoint covalent attachment on support surfaces.	Conventional supports such as cyanates, tosyl chloride and N-hydroxy-succinimide esters as carriers. Polyethylene glycol as modification agent.	The formation of several bonds between each molecule of an immobilised enzyme and the support creates a region of the enzyme in which the residues attached to the support cannot change their position during any distortion of the enzyme molecule caused by heat and organic cosolvents.	Difficult to achieve desired results.	[65]

### 3. Overview of the Enzyme Carriers Materials

Numerous literature reviews have reported on a broad range of enzyme carriers [70]. The matrix for this enzyme immobilisation must have properties such as a high surface area, high permeability, higher degree of strength, high porosity, high chemical and thermal stability, resistance and inertness towards microbial attack, insolubility in water, easy synthesis, cost-effectiveness, and being green [71–75]. The matrices or supports for this novel technology can be categorised into the following: organic and inorganic compounds. The most reported enzyme supports of organic materials are organic membrane [76], chitosan [77,78], alginate [54], resin [79], collagen [80], gelatine [81], dextran [82], starch [83], carrageenan [42], agarose [84], protein [85], cellulose [86], activated carbon [87], agar [88], and chitin [89]. Synthetic organic materials are polyvinyl alcohol [90], polyurethane foam [91], polyacrylonitrile [92], polyethylene, polypropylene membrane, and polyacrylamide [93–95]. Figure 2 depicts the enzyme carriers classified as organic, inorganic, nanomaterials, and hybrid materials.



**Figure 2.** Enzyme carriers: organic, inorganic, nanomaterials, and hybrid nanomaterials.

On the other hand, the most widely used inorganic materials are hydroxyapatite [96], gold [97,98], iron oxide [99], zirconia [100], titania [101,102], silica [103–107], silver [108,109], zinc oxide [110], alumina [111], celite [112], and inorganic clays [113]. The most recently studied enzyme carriers include graphene-based nanomaterials [114–116], metal-organic frameworks (MOFs) [117–123], and covalent organic frameworks [124–129]. The advantages of inorganic support compared with their organic counterparts are chemical, mechanical, and thermal resistance, rigidity, porosity, more reusable, and stiffness, which guarantee the stability of the novel immobilised enzyme. Organic-based support materials are more sensitive towards pH and pressure and are not suitable for bacteria and fungi growth [130].

### 3.1. Enzyme Immobilisation via Nanocarriers

Nanomaterials are referred to as materials with a minimum of one exterior dimension that vary in size from 1 to 100 nm [131–134]. Nanoparticles have recently been employed in several disciplines of biology, chemistry, physics, electronics, medicine, pharmacy, biotechnology, and chemical engineering [135–141]. Nanomaterials have distinct advantages and improved properties due to their nanoscale characteristics. The use of nanomaterials as carriers for enzyme immobilisation has shown promising results in terms of enhancing catalytic activity and stability due to their unique physicochemical properties. For instance, nanomaterials have a larger surface area, which allows for enhanced catalytic activity, mass transfer, and performance.

The unique properties of nanomaterials have led to extensive research and exploration in various fields, including enzyme immobilisation and stabilisation. Enzyme immobilisation using nanomaterials as a carrier has attracted recent attention because of good biocompatibility, ability to control the microenvironment of the immobilised enzyme, large surface area, high loading capacity, reusability, enhanced catalytic activity, good thermal stability, and easy dispersion in aqueous solution with minimal diffusion, which makes them promising nanocarriers [142–151]. Enzyme immobilisation using nanoparticles has been proven to minimise protein unfolding and enhance stability and catalytic activity [152–154].

Reducing the size of the enzyme carrier to nanosized particles could increase the efficiency and performance of the immobilised enzyme [155]. Smaller particles have a higher surface area for binding, increasing the enzyme load per unit mass of particle. The support material will allow more enzyme molecules to bind, which allows them to retain their active conformation. When nanosized porous materials are immobilised, they generate shorter routes for enzyme molecules to penetrate into the support material than bulk porous materials, resulting in decreased mass transfer resistance. Enzymes immobilised in porous nanomaterials such as nanosilica and nanographene can prevent conformational changes or denaturation, hence stabilising the enzymes [155–159]. Jain et al. [160] developed an efficient nanosilica-based immobilised *Candida rugosa* lipase using both TEOS and agrowaste rice husk ash. The adsorption and cross-linking techniques showed that an adsorption technique leads to optimum catalytic activity. The enzyme loading adsorbed on rice husk was 938 mg/g, whereas the enzyme loading adsorbed on TEOS was 925 mg/g. However, the enzymatic activity adsorbed on a rice husk precursor was 56 units/mg and 707 units/mg for the TEOS precursor.

Peiman et al. [161] synthesised a nanocatalyst containing polyamidoamine dendrimer and trypsin immobilised on magnetic nanosilica. It shows enhanced catalytic activity in the production of propargylamines in a green environment. The strength properties of silica provided a mechanically stable porous silica with an enhanced internal surface area. Due to their exceptional characteristics such as improved biomolecule compatibility, elasticity, enhanced chemical suitability, increased strength, and enhanced thermal and electrical conductivity, graphene nanoparticles have drawn a lot of interest in recent years. These qualities make them perfect for the application of nanocarriers for enzyme immobilisation [162]. Noreen et al. [163] demonstrated that functionalised graphene oxide with laccase resulted in enhanced biocatalytic capabilities and decolourisation efficiency. The immobilised laccase showed better thermal stability compared to the free enzyme at 70 °C, maintaining almost 40% of its relative activity, while the free enzyme preserved just 5.2% under the same experimental conditions. Zhou et al. [164] investigated the immobilisation of adenylate cyclase using graphene oxide. In order for the graphene oxide to match the characteristics of the enzyme, it first needed to be altered using a surface heterogeneity approach. To increase the service life of the finished product, an enzyme stabilisation approach must be employed during the immobilisation process. The interaction between the support and the adenylate cyclase can be manipulated with the flexible chain of polyethylene glycol amine and maleic anhydride that has been grafted onto the surface of the graphene oxide. Adenylate cyclase, which has been immobilised and modified graphene oxide, enhances catalytic activity by 117 times.

Zhang et al. [165] demonstrated the enzyme immobilisation of sucrose isomerase (SIase) via adsorption using graphene oxide as a nanocarrier. Some 95.3% biodegradation capability was reached using a substrate concentration of 600 g/L sucrose, 180 min at 40 °C, and pH 6.0. The temperature, pH, and storage stabilities of the immobilised ErSIase-GO were enhanced, and its activity after 10 batches was maintained at about 80% under optimum experimental conditions. Immobilised laccase had a  $K_m$  value of 29.32 mM.

### 3.2. Future Strategy Using Agrowaste Resource as a Carrier

The Industrial Revolution 4.0 (IR 4.0) is an economically viable, sustainable, green, and environmentally friendly manufacturing strategy that employs renewable resources and recyclable agrowaste materials [166–173]. Renewable agrowaste materials such as rice husk, corn cob, and eggshell are sustainable alternatives for an enzyme carrier [174–176]. Natural lignocellulosic wastes such as rice husk have gained popularity in recent years as supports for enzyme immobilisation. This is mostly because they are readily available, inexpensive, and have the potential to minimise the environmental pollution that might result from improper management [177,178]. These carriers are cost-effective and possess physiochemical properties such as a large surface area, high stiffness, high tensile strength, excellent adsorption capacity, high strength, zero deformation, and low density

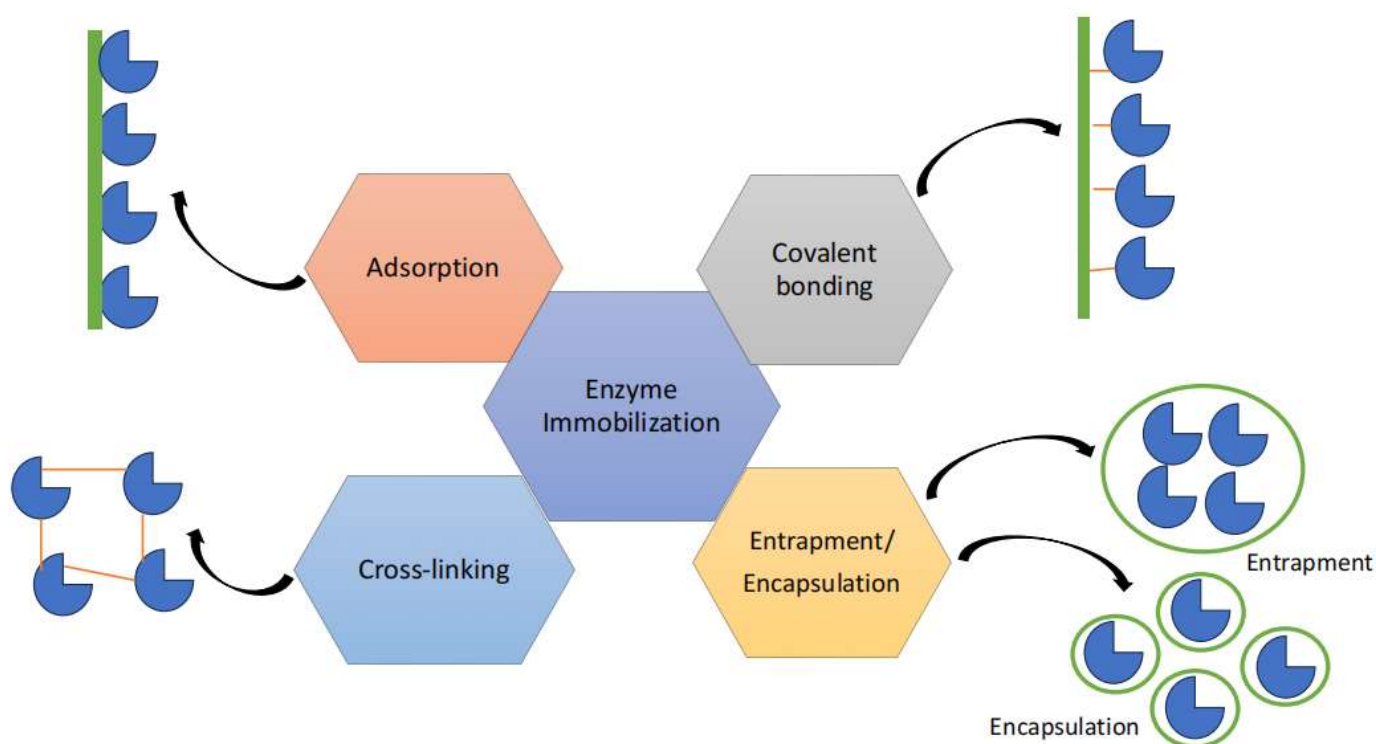


and porosity; reactive functional groups are excellent for supporting biocatalysts [179]. This approach will drastically lower the cost of enzymes, which is one of the main limitations of employing a biocatalyst in industrial applications [177].

Enzyme immobilisation using an agrowaste carrier and its application is still limited. However, recent studies have shown promising results in utilising sustainable materials such as agricultural residues, industrial waste, and waste biomass for enzyme immobilisation. These alternative materials not only provide a cost-effective and eco-friendly solution but also offer potential advantages in terms of enzyme stability, catalytic activity, and reusability. Spennato et al. [180] studied lipase immobilisation by covalent bonding with agrowaste rice husk as a carrier. The resulting immobilised lipase was mechanically stable, recyclable, and suitable for use in various hydrophobic mediums. Yassin and Gad [181] investigated the covalent immobilisation of horseradish peroxidase using the packaging waste of expanded polystyrene foam (EPS)–polydopamine as a carrier. The horseradish peroxidase (HRP) was covalently immobilised using a Michael addition or Schiff base reaction with the polydopamine layer as a reactive framework. The loading capacity was approximately 46%. At a strong alkaline condition (pH 10), the immobilised peroxidase retained 53% of its activity. The immobilised peroxidase observed a higher catalytic activity of approximately 95% compared to 56% of free peroxidase at elevated temperature (60 °C).

Brown onion skins, egg shell membrane, nanosilica rice husk, guava seed biochar, graphene oxide grape seed biochar, tamarin seed activated carbon, activated carbon derived from *Prosopis juliflora* bark, and biochar derived from apple branches area are reported to be used as enzyme agrowaste carriers. Kumar and Pundir [17] used glutaraldehyde to covalently immobilise lipase on the onion membrane. Immobilised lipase remains at 63.6% of its catalytic activity. When kept at +4 °C, the immobilised lipase was utilised up to 100 times in two months without a substantial loss in activity. Kessi et al. [18] demonstrated that the bound and free enzymes are comparable in their catalytic activity. Immobilised  $\beta$ -galactosidase is more stable and can also be utilised multiple times. In the presence of skim milk serum, immobilised  $\beta$ -galactosidase may hydrolyze lactose. Utomo et al. [19] investigated that the contact time and agitation speeds have no effect on the percentage of cellulase immobilisation on rice husk silica. Based on its activity, the optimal contact duration and agitation speed for immobilised cellulase were 15 min and 100 rpm. When compared to the first cycle, immobilised cellulase activity in cycles II and III reduced to 75.2% and 58.8%, respectively.

On the other hand, lipase immobilisation via adsorption on guava seed charcoal was investigated by Almeida et al. [20]. The optimal lipase loading was 0.15 g enzyme/g support, with a hydrolytic activity of 260 U/g and a 54% immobilisation yield. Under diverse reaction conditions, the products of a transesterification process catalysed using immobilised lipase yielded the highest yield at 40 °C. Thiyagarajan et al. [21] investigated laccase immobilisation on activated carbon nanotubes produced from *Prosopis juliflora* bark to enhance its stability, reusability, and magenta dye adsorption efficiency. The experimental condition was optimised using a response surface methodology (RSM)-based Box–Behnken design (BBD), and the maximum MD adsorption was about 95% at 120 min. Zou et al. [27] synthesised immobilised laccase using biochar derived from cellulase hydrolysis apple branch powder. Immobilised laccase demonstrated enhanced stability against pH ranges, thermal, storage duration, and operation. Figure 3 shows an illustration of the strategies of enzyme immobilisation using agrowaste nanocarriers. Table 2 shows the recent methods for immobilising enzymes using innovative agrowaste nanocarriers and their results.



**Figure 3.** An illustration of enzyme immobilisation methods using agrowaste nanocarrier. Clarification: = enzyme; or = carriers; = linker.

**Table 2.** Recent strategies for enzyme immobilisation using novel agrowaste nanocarriers and its distinguished outcome.

Enzymes	Immobilisation Methods	Agrowaste Carriers	Merits	References
Lipase	Cross-linking	Brown onion skins	The catalytic activity of immobilised lipase has retained 63.6%. It could be reused more than 100 times for 60 days.	[182]
$\beta$ -galactosidase	Adsorption	Egg shell membrane	The results demonstrate a similarity between the bound and free enzymes as well as the stability and reusability of the immobilised $\beta$ -galactosidase	[183]
Cellulase	Adsorption	Nanosilica rice husk	The immobilised cellulase retained its catalytic activity. It could be reused many times, with catalytic activity decreasing from 75.5 to 58.8% in the third cycle.	[184]
Lipase	Adsorption	Guava seed biochar	The optimal BCL loading was found to be 0.15 g enzyme/g support with 260 U/g of hydrolytic activity and 54% immobilisation yield. Under numerous reaction conditions, the highest yield of transesterification products was achieved at 40 °C.	[185]
Lipase	Adsorption	Activated carbon derived from <i>Prosopis juliflora</i> bark	The parameters were optimised using response surface methods, and the maximum magenta dye adsorption using immobilised lipase was about 95% at 120 min.	[186]
Pepsin	Adsorption and Covalent Bonding	Biochar derived from pupunha palm waste	The immobilised enzyme retains its biological activity up to seven times.	[187]

Table 2. Cont.

Enzymes	Immobilisation Methods	Agrowaste Carriers	Merits	References
Lipase	Adsorption	Activated carbon derived from tamarin seed	It can retain 86% of its catalytic activity after five times reuse.	[188]
Lipase	Adsorption	Graphene oxide grape seed biochar	It can retain 60% of its catalytic activity for more than five times reuse.	[189]
Lipase	Adsorption	Nanosilica rice husk	It can retain 85% to 90% of its initial activity after nine cycles.	[190]
Laccase	Adsorption, Covalent Bonding	Hydrocolloid and fibre industry waste	The adsorption capacity for removal of organic pollutants was enhanced using immobilised laccase compared to without laccase.	[191]
Laccase	Adsorption	Microporous biochar derived from apple branches	The biodegradation rate of immobilised laccase for norfloxacin, enrofloxacin and moxifloxaci after a 48-h reaction were 93.7%, 65.4%, and 77.0% at pH 4 and 40 °C, respectively. These values were 1.2, 1.3, and 1.3 times higher than those of MBC under the same experimental condition.	[192]
Laccase	Adsorption	Biochar derived from agrowaste	The immobilised laccase demonstrated enhanced pH tolerance, and thermal and storage stability compared to free laccase.	[192]
Laccase	Adsorption	Biochar derived from corn cob	The optimum catalytic activity of immobilised enzyme was found at pH 4.0 and 25 °C. The immobilised enzyme retained 50% of its initial activity after 30 days of storage duration.	[193]
Lipase	Adsorption	Palm waste-activated carbon	The catalytic activity of immobilised lipase was enhanced compared to free lipase. The optimum condition of immobilised lipase was 0.5 (NaOH (g)/palm raceme (g)), 150 min, and 400 °C for carbonisation.	[194]
Laccase	Adsorption	Activated biochar derived from agrowaste	Immobilised laccase had significantly higher catalytic activity than free laccase throughout a pH range of 3.5 to 6.5 and a temperature range of 30 to 60 °C. After 5 h at 55 °C, the immobilised laccase retained 50% of its catalytic activity. It could be reused 6 times with and kept above 60% of its catalytic activity, compared to free laccase at about 40%.	[195]
Laccase	Covalent bonding and adsorption	Eggshell	The study found that immobilising periodate-oxidised laccase on NiCl <sub>2</sub> -pretreated eggshell membrane was the best method with an immobilised activity of 1300 U/Kg and a 30% residual activity after 6 reuses. The covalent method with glutaraldehyde was the best for the enzyme-dropping protocol, with an immobilised activity of 3500 U/Kg and a 45% residual activity after 6 reuses.	[196]
Peroxidase	Covalent bonding	Rice straw biochar	Peroxidase immobilisation on functionalised biochar demonstrated three times higher catalytic activity and improved stability against extreme pH and temperature.	[197]

#### 4. Future Recommendations and Challenges

There are several recommendations for the future direction of enzyme immobilisation:

1. Nanotechnology
  - Recent developments in nanotechnology are believed to have a considerable impact on enzyme immobilisation. For instance, nanoparticles have unique features, including a large surface area and improved catalytic activity. These materials can provide the optimum environment for enzyme immobilisation, enhancing stability and efficiency.
2. Advance in bioinformatics
  - Bioinformatics tools and enzyme engineering advancements, such as directed evolution and rational design, could contribute to the development of enzymes with improved characteristics, such as increased stability, substrate selectivity, and catalytic efficiency for immobilisation. We can improve performance and stability by designing enzymes, specifically for immobilisation and stabilisation.
3. Multienzymatic systems
  - Methods for immobilising numerous enzymes or multienzymatic systems should be investigated, and effective multienzyme systems for complex processes should be designed.
4. Three-dimensional printing technology in customise complexed immobilised enzyme
  - The use of 3D printing technology should be explored to precisely organise enzymes within immobilisation matrices, allowing for complex and customised designs.
5. Environmentally friendly and renewable carriers
  - We emphasise creating environmentally friendly and sustainable immobilisation procedures, taking into account factors such as the utilisation of renewable carriers and the reusability of immobilised enzymes.
6. Bioelectrochemical systems
  - Enzyme immobilisation in bioelectrochemical processes is relatively new, and it might lead to the development of more sustainable and efficient technologies for energy, environmental, and healthcare purposes. These studies involve the interactions between enzymes and electrode surfaces.
7. Scaling-up
  - We should work on the industrial-level manufacturing of immobilised enzymes, taking into account factors such as profitability, scaling, stability, and catalytic activity efficiency under industrial settings.

Although enzyme immobilisation has many advantages, there are challenges that need to be addressed. Several challenges remain still to be tackled, such as the following:

1. Mass transfer limitations:
  - Internal and external mass transfer limitation will be improved by enhancing the surface area and altering the geometry or shape of the carrier's enzyme,
2. Enzyme denaturation and inactivation:
  - Enzymes are delicate and easily denatured under harsh micro and macro conditions.
3. Complexity, cost, and scalability:
  - We should focus on the synthesis and development of simple, low-cost, large-scale, improved catalytic activity and stability for large-scale industrial applications.
4. Uniformity and reproducibility:
  - Immobilised enzymes frequently suffer reproducibility issues. This is owing to the fact that immobilised enzymes can undergo conformation changes alter and lose catalytic activity over time. These difficulties can be caused by enzyme denaturation or changes in the microenvironment around the immobilised enzyme.

- Strengthen the consistency and repeatability of enzyme immobilisation methods to ensure identical and consistent results for each batch.
5. Unstable in harsh environmental conditions:
    - The stability of immobilised enzymes should be boosted, and their catalytic activity should be retained under harsh environmental conditions, including extremely low and high temperatures, pH, organic solvents, and inhibitors.
  6. Regeneration and reusability issues:
    - We should develop enzyme immobilisation methods that promote regeneration and reusability of immobilised enzymes while retaining activity even after several cycles of reaction.
  7. Long-term storage stability issues:
    - We should develop methods to improve the long-term storage stability of immobilised enzymes, especially for applications such as biosensors, biofuel cells, and bioreactors.

## 5. Conclusions

Enzyme immobilisation is a key technology in catalysis, biosensing, and bioprocessing, offering higher catalytic activity, improved thermal and storage stability, reduced cost, and sustainability. Methods like physical adsorption, covalent binding, and entrapment/encapsulation offer numerous advantages in terms of enzyme activity, operational stability, reusability, and ease of chemical operation. The optimal method of immobilisation depends on the enzyme, the substrate, the carrier, the method of immobilisation, and the whole desired process. It is crucial to take into account the characteristics of each enzyme in order to determine the most effective immobilisation method. Factors such as enzyme stability, activity, and desired reaction conditions should be taken into account when selecting the appropriate immobilisation approach. The choice of carrier material should be carefully considered to ensure compatibility with both the enzyme and the desired process conditions. The commercialisation of immobilised enzymes using nanomaterials and renewable carriers is still in its early stages, but their development holds great promise for further advancements in catalysis and bioprocess. The commercialisation of immobilised enzymes using agrowaste nanocarrier is expected to gain momentum in the near future. This review provides a comprehensive overview of the different methods used for enzyme immobilisation, including physical adsorption, covalent binding, and entrapment/encapsulations. It highlights the recent advancements in novel carriers such as nanoparticles and agrowaste materials, which offer improved enzyme catalytic activity, stability and reusability.

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