Enzyme Immobilization Techniques

Subjects: Biochemical Research Methods

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Researchers have explored the technique of enzyme immobilization as a means to overcome limitations that free enzymes encounter, including reduced performance, high costs, and impracticality for large-scale applications. Enzymatic treatment offers a sustainable and eco-friendly alternative to conventional physicochemical treatment methods, such as adsorption, coagulation, and advanced oxidation processes. Free enzymes are biodegradable, highly efficient, and selective biological catalysts that can operate under mild conditions, thus reducing energy consumption and minimizing the generation of harmful byproducts.

Keywords: enzyme immobilization ; wastewater treatment

1. Introduction

Environmental pollution has become a major global concern, with a wide range of contaminants, such as phenolic compounds and organic dyes, posing significant threats to water quality and ecosystem health [1][2][3][4]. The increasing release of these toxic and recalcitrant substances into the environment, originating from various industrial wastewater streams such as textile, pharmaceutical, and petrochemical industries, has caused widespread concern [5][6]. Contaminated water sources can lead to adverse effects on aquatic life and have potentially detrimental consequences for human health, as these pollutants may exhibit carcinogenic, mutagenic, or teratogenic effects [7]. In particular, understanding the effect of organic dyes and phenolic compounds on human health is crucial for the development of effective policies and remediation strategies to minimize the risks associated with these contaminants [8]. As the demand for clean water continues to increase due to population growth and industrialization, there is an urgent need to develop effective, efficient, and sustainable strategies for environmental remediation, focusing on the removal of these pollutants from wastewater [8][9][10].

Numerous conventional techniques have been employed to treat phenolic wastewater and organic dyes, each with its own advantages and disadvantages. For instance, adsorption is simple, flexible, and can be highly efficient with low capital cost, but the performance is adsorbent-dependent, and some adsorbents can be expensive [11][12][13][14][15][16][17]. Distillation, while effective, can be energy-intensive and may not be suitable for all types of contaminants [18][19][20][21]. Chemical oxidation can be highly effective, but it often involves the use of harsh chemicals that can be harmful to the environment [22][23][24]. Extraction is a fast and simple process, but it requires highly selective solvents, which can be expensive and harmful to the environment [25][26][27][28]. Membrane separation is easy to operate with high selectivity, but it can be expensive and suffers from issues such as membrane fouling [29][30][31][32]. Photocatalytic oxidation is a promising technique, but it often requires specific conditions to be effective [33][34][35][36].

Given these limitations of conventional techniques, there has been growing interest in environmental protection through the effective treatment of polluting streams [37][38][39][40], the utilization of sustainable and biodegradable materials [41][42][43][44][45][46][47][48], and the application of microorganisms for the biological treatment of wastewater contaminated with organic dyes, and phenolic compounds have also been explored [49][50]. Microorganisms utilize various enzymatic systems for the oxidative transformation of organic molecules, including laccases, ligninases, tyrosinases, monooxygenases, and dioxygenases [51]. Flavoenzymes, known as azoreductases, are present in both microorganisms and higher eukaryotes and are involved in the detoxification and biotransformation of azo dyes [52]. Furthermore, microorganisms utilize specific intracellular enzymes, namely oxidoreductases, to catalyze the metabolism of phenolic compounds [53]. These enzymatic systems offer several advantages over conventional techniques, including cost-effectiveness, sustainability, and the ability to operate under mild conditions [54][55][56]. This highlights the critical function of enzymes in the biodegradation processes of various organic pollutants, demonstrating the potential of harnessing these enzymatic systems for efficient and eco-friendly pollutant removal strategies [56]. As a result, a new approach has arisen in recent times where extracellular

enzymes are utilized instead of whole microbial cells for the remediation of wastewater contaminated with organic substances.

Enzymes act as highly effective biological catalysts that enable specific reactions to occur. The lock and key model or the induced fit model can be used to explain their efficiency. By reducing the activation energy and stabilizing the transition state, enzymes enhance the reaction rate [57][58]. Enzymes possess desirable qualities such as high efficiency, high selectivity, and the ability to operate under milder conditions compared to other chemical catalysts. Enzymes offer a cost-effective advantage in that they operate under mild conditions, eliminating the need for expensive equipment that would otherwise be required for chemical catalysts to achieve extreme conditions such as high pressure or temperature. Their natural origin also makes them environmentally friendly due to their biodegradability and low environmental impact.

There has been growing interested in employing enzymes for the treatment of dye wastewater, and previous research has examined the use of various enzymes, including soybean peroxidase [59][60], horseradish peroxidase (HRP), lignin peroxidase (LiP) [61], and laccase [62][63], for their potential in treating dyes. Peroxidase, a member of the oxidoreductase enzyme family, can enable the oxidation of diverse substances in the presence of an oxidizing agent like chlorine, hydrogen peroxide, and potassium permanganate. A significant use of peroxidase is its ability to degrade aromatic compounds, especially synthetic dyes. This occurs when they are decomposed into individual components and the oxidative polymerization of phenolic compounds is triggered, leading to the creation of insoluble polymers [58][64]. Hydrogen peroxide plays a crucial role in the catalytic cycle of peroxidase enzymes. The reaction begins with the reaction between the Fe(III) state of peroxidase and hydrogen peroxide, leading to the formation of a high-oxidation-state intermediate consisting of a cation radical based on porphyrin and an Fe(IV) oxo ferryl center [65]. After the initial oxidation, the process consists of two reduction steps that bring the peroxidase back to its original state, compound II, with the production of free radicals. These free radicals then undergo polymerization. However, a high concentration of hydrogen peroxide may inhibit the process, leading to a decrease in enzymatic activity [66].

The application of enzymes for dye wastewater treatment at an industrial scale is often hindered by various limitations such as elevated production expenses, reduced long-term operational stability, restricted reusability, and limited shelf life after the initial use [59][67]. In their crude form, enzymes may exhibit limited catalytic activity because of their vulnerability to inhibition, particularly in the case of complex dye wastewater [68]. Heavy metals can have a detrimental effect on enzymatic activity, as different enzymes show varying degrees of sensitivity to these substances [58]. In certain cases, heavy metals, such as mercury, can react with the reactive groups present in enzymes and render them incapable of catalyzing further reactions [69]. As the complexity of wastewater effluent increases, it is expected that enzyme activity will decline, further underscoring the challenges associated with the use of enzymes in the treatment of wastewater [70][71].

In recent years, the use of free enzymes has gained considerable attention among the various proposed methods for removing phenolic compounds [72][73][74][75]. Enzymatic treatment offers a sustainable and eco-friendly alternative to conventional physicochemical treatment methods, such as adsorption, coagulation, and advanced oxidation processes [76][77]. Free enzymes are biodegradable, highly efficient, and selective biological catalysts that can operate under mild conditions, thus reducing energy consumption and minimizing the generation of harmful byproducts [78][79].

Researchers have explored the technique of enzyme immobilization as a means to overcome limitations that free enzymes encounter, including reduced performance, high costs, and impracticality for large-scale applications [56][80][81]. Enzymes have limited operational stability, which can negatively impact their catalytic efficiency. Factors like temperature, pH, and exposure to harsh conditions or solvents can lead to enzyme denaturation, degradation, or aggregation, thereby limiting their effectiveness [82]. Enzyme immobilization can improve the stability of enzymes as it offers a physical support system that safeguards them against destabilizing agents while maintaining their original structure [80][83]. The recovery and reuse of free enzymes after a reaction can be time-consuming and costly, but enzyme immobilization allows for easy separation from the reaction mixture and enables reuse for several reaction cycles, thereby decreasing the overall costs of enzyme usage [84][85][86]. In some reactions, free enzymes can experience reduced catalytic activity due to mass transfer limitations, substrate and product inhibition, or poor substrate solubility in water. Immobilization can overcome these challenges by creating custom biocatalytic systems with improved mass transfer properties, enhanced enzyme-substrate interactions, and optimized reaction conditions [54]. Moreover, free enzymes may exhibit low selectivity in certain reactions, especially when working with chiral compounds or complex substrate mixtures. Immobilization has the potential to enhance selectivity by enabling precise control over enzyme orientation and creating a microenvironment that promotes selective catalysis [87][88].

According to Nguyen et al. [89], immobilized enzymes are more effective in eliminating phenolic compounds than free enzymes due to the synergistic effect of enzymatic reactions and pollutant adsorption on the solid support. While not

examining the competition between the adsorption of pollutants and products or the influence of product adsorption on enzyme activity, previous research has demonstrated that the adsorption capacity of the support medium deteriorates after multiple applications, even in the absence of enzymes. This suggests that irreversible adsorption of pollutants or products could be involved in the overall process, particularly when immobilized enzymes are recycled. Nguyen et al. [89] suggested that addressing the removal of irreversibly adsorbed pollutants or products could potentially enhance the effectiveness of the immobilized enzyme system.

There is a broad range of nanomaterials that are widely employed for the purpose of enzyme immobilization. This includes but is not limited to metal oxides [90], carbon dots [91], covalent organic frameworks (COFs) [92], graphene [93], CNTs [94], and MOFs [95]. Each of these nanomaterials possesses unique properties that make them suitable for enzyme immobilization. They often exhibit a high surface area to volume ratio, excellent conductivity, good chemical stability, and strong adsorption capabilities, which make them advantageous in improving the performance of immobilized enzymes [96].

Metal oxides like titanium dioxide and zinc oxide offer robustness, chemical stability, and biocompatibility, making them useful for enzyme immobilization [90][97]. Carbon dots, with their superior optical properties and biocompatibility, also have applications in this area [91]. COFs, due to their designable structures, large pore size, and high surface area, provide ideal platforms for enzyme immobilization [92]. However, this review specifically focuses on the unique advantages of graphene, CNTs, and MOFs for enzyme immobilization. Graphene and its derivatives, such as graphene oxide (GO), are widely used for enzyme immobilization due to their high surface area, excellent thermal and electrical conductivity, and strong π-π stacking interactions, which allow effective enzyme adsorption and retention of their bioactivity [83][98]. CNTs offer similar benefits, with additional advantages coming from their tubular structure, which provides a protective environment for enzymes, enhancing their stability and reusability [99][100]. MOFs, with their highly ordered structures and large surface areas, offer unique possibilities for enzyme immobilization. Their pore size, shape, and functionality can be finely tuned, allowing the accommodation of a wide range of enzymes while preserving their activity and stability [101][102].

2. Enzyme Immobilization Techniques

Enzyme immobilization is a technique to improve enzyme stability and reusability while maintaining their activity. It refers to the physical or chemical confinement of enzymes in a distinct phase different from the substrate's phase [103]. These techniques can be classified into two broad categories: physical and chemical methods [103][104].

2.1. Physical Techniques

Physical immobilization, as the earliest form of immobilization, only involves physical interactions. In this method, neither the immobilizer nor the immobilization agent is changed, linked, or modified. This technique includes encapsulation, entrapment, and adsorption. These processes do not necessitate a covalent bond between the enzyme and the support, therefore maintaining the enzyme's native structure [105]. Adsorption involves enzymes interacting with a support material through forces such as hydrophobic interactions or salt bridges, while entrapment is a technique where enzymes are confined within gels or fibers using covalent or non-covalent bonds. Similarly, encapsulation secures enzymes within semi-permeable capsules, allowing for the movement of small substrates or products while restricting the migration of larger enzymes [104][105][106].

2.1.1. Adsorption

Enzymes can be adsorbed onto support materials through interactions such as hydrophobic forces and salt bridges. Enzyme adsorption onto the support physically can be achieved by immersing the support material in the enzyme solution or by drying enzymes onto electrode surfaces. This immobilization method protects the adsorbed enzymes from factors such as proteolysis, aggregation, and interaction with hydrophobic surfaces [107]. Scientists have utilized eco-friendly materials as enzyme supports to promote sustainable practices. For instance, coconut fibers can retain high amounts of water and have strong cation exchange properties, microcrystalline cellulose has a strong binding capacity, and kaolin offers good enzyme retention through micro/mesoporous materials and chemical acetylation with thiol functionalization and large surface areas that are suitable for redox reactions [108][109][110][111][112][113]. Silanized molecular sieves have been found to be an effective support for enzyme adsorption, owing to the presence of silanols on the surface of the pores that allow for enzyme immobilization through the process of hydrogen bonding [114]. Modifications to the current support materials could potentially enhance enzyme immobilization. Prior investigations have delineated the water activity patterns of polypropylene hydrophobic granules-bound lipase, notably Accurel EP-100 [115]. It was observed that reducing the particle size of Accurel has a positive effect on reaction rates and enantiomeric ratios during biocatalysis [116].

To improve both process control and the cost-effectiveness of production, the immobilization of Yarrowia lipolytica lipase on supports like octadecyl-sepabeads and octyl-agarose through physical adsorption has been explored. As a result of this process, there were significant improvements in yields and a tenfold increase in stability when compared to free lipase. Octadecyl-sepabeads, which are hydrophobic in nature, enhance the affinity between the enzyme and support, explaining this observation [117]. After being adsorbed onto biodegradable poly (3-hydroxybutyrate-co-hydroxyvalerate), Candida rugosa lipase was able to retain 94% of its activity after four hours at 50 °C and could be reused for up to 12 cycles [118]. The supports were selected due to their flexible and less ordered nature when compared to polyhydroxybutyrate. Byssus threads activated with 1,4-butanediol diglycidyl ether provided a suitable matrix for immobilizing urease, leading to enhanced pH stability and maintaining 50% of the activity of the enzyme under dry conditions [119]. In recent years, biocompatible mesoporous silica nanoparticles (MSNs) have gained attention as an environmentally sustainable support for biocatalysis. The use of these supports not only reduces production costs but also avoids ethical concerns. Due to their durability and effectiveness, MSNs have been applied in energy-related biocatalytic processes [120]. **Table 1** presents the benefits, challenges, and solutions for overcoming the limitations of the adsorption technique.

Table 1. Common immobilization techniques, their advantages and disadvantages, and suggested approaches to overcome limitations.

Immobilization Technique	Advantages	Drawbacks	Approaches to Address the Limitations	Ref.
Adsorption	• Prevention of proteolysis • Full activity retention	• Non-targeted adsorption • The expense of affinity binding • The activity is affected by a slight shift in the reaction conditions • The leaching of enzymes	• Using a blocking agent to reduce interactions that aren't specific • Specific pH for the charge difference between the silica support and the enzyme • Pore size decrease following adsorption	[121] [122] [123]
Entrapment	• Moderate preparation circumstances • Prevents direct contact with the environment outside	• Limited mobility on mass transfer • Leakage is the result of fewer physical restraints	• Exact pore size selection based on enzyme size • Further covalent fusion	[124] [125] [126]
Encapsulation	• Maintenance of enzymatic activity over prolonged periods • Easy passage of small substrate molecules • Large enzymes confined within the capsules	• Difficulties in ensuring optimal diffusion of substrates and products • Maintaining the structural integrity of the capsules under operational conditions	• Development of capsules with improved stability, selectivity, and permeability • Advances in materials science for better encapsulation materials and methods, such as 3D capsules	[126] [127] [128] [129]

Immobilization Technique	Advantages	Drawbacks	Approaches to Address the Limitations	Ref.
Covalent binding	• Reduced limitations of mass transfer • Improved storage and stability of reaction • Stronger bonding	• Specific binding site • Denaturation of the enzyme's active site • Irreversible binding	• Support and enzyme modification • Specific binding site	[130] [131] [132]
Cross-linking	• Aggregates may experience increased activity • Recyclability, higher loading capacity, and total activity retention	• The cross-linking matrix's fragility • Agents that precipitate conflict • The pure enzyme is necessary for cross-linking enzyme crystals	• The ideal aggregate size determined by the cross-linker-to-enzyme ratio • Stabilizing components for the structure • Using cross-linking enzyme crystals for enzymes that haven't been fully purified	

2.1.2. Entrapment

Entrapment involves confining enzymes within gels or fibers using covalent or non-covalent bonds [133]. Effective entrapment has been realized with hybrid carriers made of alginate, gelatin, and calcium, which prevent the enzyme from leakage and offer increased mechanical stability [134]. The implementation of nanostructured materials in enzyme immobilization, such as pristine materials and electrospun nanofibers, which are produced through a method known as electrospinning, has significantly impacted the field. Mesoporous silica entrapment has recently emerged as a highly promising technology in fields such as biomedicine, fine chemistry, biosensors, and biofuels. This is largely due to the material's unique properties, including a large surface area, uniform pore distribution, adjustable pore size, and high adsorption capacity. These features enable mesoporous silica to serve as an effective support material for various applications [135]. Lipase and magnetite entrapment of nanoparticles simultaneously within biomimetic silica has been shown to increase activity with various silane additives [136]. In the meantime, the selective binding and carrying properties of sol–gel matrices with supramolecular calixarene polymers have been used to entrap C. rugosa lipase [137][138]. In **Table 1**, entrapment's advantages, disadvantages, and strategies to tackle its limitations are summarized.

2.1.3. Encapsulation

The method of encapsulation immobilization entails the confinement of a variety of biomolecules within distinct polymeric structures [139]. This process shares similarities with entrapment, as both techniques permit enzymes and cells to exist freely within a solution while remaining in a controlled environment. Encapsulation aims to secure delicate enzymes and cellular solutions within small vesicles with porous barriers, preventing larger enzymes from exiting or entering the capsules, while smaller substrates or products can traverse the semi-permeable barrier with ease [127]. This method allows for the preservation of biological systems within a thin protective film, preventing direct environmental exposure that could negatively affect the performance of the biocatalysts, hence, enabling the prolonged activity of these biocatalysts [140]. Various supportive materials, such as cellulose nitrate and nylon, are employed in the production of microcapsules that range in size from 10 to 100 μm [141]. Furthermore, the process of ionotropic gelation of alginates and nanoporous silica-based sol–gel glasses has proven its efficacy in the field of enzyme encapsulation.

The simplicity of the encapsulation process distinguishes it, and advancements in material sciences have led to the improvement of this method, with benefits such as increased morphological stability, customizable physicochemical permeability, and reduced enzyme leakage [141]. The technique also offers the potential for co-immobilization, allowing for the possibility of immobilizing enzymes in any combination as required. Nevertheless, the method is not without its limitations. For example, issues related to diffusion can be significant, with the risk of membrane rupture if reaction products accumulate rapidly [128]. **Table 1** provides a summary of the benefits, limitations, and strategies to overcome the challenges associated with the encapsulation method.

2.2. Chemical Techniques

Chemical methods involve the formation of strong covalent bonds between the enzyme and the support, leading to higher stability and reusability. Chemical techniques include covalent binding, cross-linking, and affinity immobilization. Covalent binding attaches enzymes to supports through covalent bonds formed with specific amino acids in the enzyme's side chains [124]. Cross-linking forms covalent bonds between enzyme molecules using bifunctional or multifunctional agents. Affinity immobilization is a technique that utilizes the enzyme's specific binding properties to support materials under different physiological conditions [104][142].

2.2.1. Covalent Binding

Enzymes can be attached to supports through covalent binding, which relies on specific amino acids in the enzyme's side chains, such as arginine, aspartic acid, and histidine. The effectiveness of this process is largely determined by the reactivity and efficiency of the functional groups present in the support, such as imidazole, indolyl, and phenolic hydroxyl [133]. Utilizing surfaces modified with peptides for enzyme immobilization leads to enhanced specific activity and stability of the enzymes, as well as the regulated orientation of the proteins [143]. Using CNBr-activated agarose and CNBr-activated sepharose, which have carbohydrate moieties and glutaraldehyde as a spacer arm, is one method for covalently attaching enzymes to supports. According to studies, this immobilization strategy has proven to give the linked enzymes thermal stability [117][144]. Through covalent enzyme attachment, silica gel carriers modified with silanization and SBA-15 supports with Si-F-lined cage-like pores created highly stable and hyperactive biocatalysts [145]. The enhanced half-life and thermal stability of enzymes have been achieved via covalently attaching them to various supports such as mesoporous silica and chitosan [135][144]. Covalently linking enzymes to electrospun nanofiber leads to improved residual activity as a result of greater surface area and porosity. The implementation of nanodiametric supports has revolutionized biocatalyst immobilization [146][147][148][149][150]. Alcohol dehydrogenase was covalently bound to attapulgite nanofibers (hydrated magnesium silicate) due to their thermal endurance and varying nanosizes [151]. Cross-linked enzyme aggregates have been developed by precipitating enzymes from aqueous solutions using organic solvents or ionic polymers [152]. The pharmaceutical industry has found covalent binding to magnetic nanoclusters to be useful in achieving varied orientations of immobilized enzymes. This approach has resulted in enhanced operational stability, durability, and reusability, making it a promising technique for enzyme immobilization [153]. One important function of cross-linking agents in enzyme immobilization is to maintain the enzymes' structural and functional integrity. Glutaraldehyde is a commonly used bifunctional cross-linker that can form stable covalent bonds both within and between enzyme subunits, thereby preserving the enzyme's activity and structure. It is also soluble in aqueous solvents, making it a convenient option for use in enzyme immobilization processes. **Table 1** outlines the advantages, drawbacks, and approaches to address the limitations associated with covalent binding.

2.2.2. Cross-Linking

Cross-linking is a method of immobilizing enzymes that do not require a support material and results in irreversible binding, preventing the enzyme from leaking into the substrate solution [142][154][155]. This immobilization technique, referred to as carrier-free immobilization, allows the enzymes to act as their carrier, thus resulting in a pure enzyme product and avoiding the drawbacks of using carriers [125][156]. The addition of carriers for enzyme immobilization may result in a decrease in activity, as the presence of non-catalytic components, referred to as ballast, can account for a significant proportion of the total mass, ranging from 90% to over 99%, ultimately leading to reduced space-time yields [152][156] and increased costs [152].

Cross-linking is a process of forming covalent bonds between enzyme molecules using bifunctional or multifunctional agents. One of the commonly used cross-linking agents is glutaraldehyde, owing to its affordability and large-scale availability [125][157]. For several decades, glutaraldehyde has been extensively utilized as a cross-linking agent to generate intermolecular cross-links between proteins, such as enzymes. The cross-linking of enzymes occurs through a reaction with free amino groups of lysine residues on neighboring enzyme molecules. This results in the formation of oligomers or polymers through both inter- and intramolecular aldol condensations, with the specific type of cross-linking dependent on the pH [152][158].

Cross-linked enzyme aggregates (CLEAs) are formed by precipitating enzymes with ammonium sulfate, acetone, or ethanol and then treating the aggregates with a cross-linking agent [125]. There are three methods for immobilizing enzymes, which are: (1) the blending of prepolymer and photosensitizer followed by gelling under near-UV radiation, (2) the freezing of enzyme-containing monomer solution into beads and subsequently polymerizing by gamma radiation, and (3) chemical polymerization via the combination of enzymes with acrylamide monomer and a cross-linking agent in a buffered aqueous solution. [159]. Lately, nanodiametric supports have induced significant advancements in biocatalyst

immobilization [56][96][160][161]. The cross-linking immobilization of enzymes on electrospun nanofibers has been shown to improve residual activity, ascribed to the larger surface area and porosity of the substrate. CLEAs were employed to immobilize lysozyme on electrospun chitosan (CS) nanofibers, yielding a durable antibacterial material that can be used continuously [162]. **Table 1** provides an overview of the merits, drawbacks, and methods to address the challenges related to the cross-linking technique.

2.2.3. Affinity Immobilization

The affinity immobilization of enzymes involves utilizing their specific binding properties to support materials under different physiological conditions. There are two approaches to achieving this: first, by linking an affinity ligand specific to the target enzyme to the matrix, or second, by attaching the enzyme to a molecule that develops an affinity for the matrix [163]. The use of affinity adsorbents has not only been limited to the purification of enzymes but has also been extended to their simultaneous purification [164]. Sophisticated affinity matrices like chitosan-modified porous silica beads that are stable in alkali environments and multilayered concanavalin A attached to agarose are capable of immobilizing greater amounts of enzymes leading to better stability and efficiency [165][166]. The technique of bio affinity layering is an improvement over affinity immobilization, and it can significantly increase the capacity for enzyme binding and reuse. The non-covalent interactions, such as van der Waals forces, coulombic forces, and hydrogen bonding, among others, are utilized for this purpose [166][167].

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