

Review

# Current Status and Future Perspectives of Supports and Protocols for Enzyme Immobilization

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**Abstract:** The market for industrial enzymes has witnessed constant growth, which is currently around 7% a year, projected to reach \$10.5 billion in 2024. Lipases are hydrolase enzymes naturally responsible for triglyceride hydrolysis. They are the most expansively used industrial biocatalysts, with wide application in a broad range of industries. However, these biocatalytic processes are usually limited by the low stability of the enzyme, the half-life time, and the processes required to solve these problems are complex and lack application feasibility at the industrial scale. Emerging technologies create new materials for enzyme carriers and sophisticate the well-known immobilization principles to produce more robust, eco-friendlier, and cheaper biocatalysts. Therefore, this review discusses the trending studies and industrial applications of the materials and protocols for lipase immobilization, analyzing their advantages and disadvantages. Finally, it summarizes the current challenges and potential alternatives for lipases at the industrial level.

**Keywords:** lipase; immobilization; support; protocol; biocatalysis; co-immobilization; biocatalyst; enzyme; novel techniques



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## 1. Introduction

The continuous interest of the market and the academy in biotechnological alternatives to the waste of byproducts and power attached to conventional industrial processes are opening and exploring new study fields in biocatalysis. However, there are still many potentials to be exploited in this subject [1–4]. The enzyme market is growing by around 7% a year, relying on new ways of cheapening their use and the well-known advantages of mild reaction conditions, specificity, reduced byproduct formation, product separation, biodegradability, and high efficiency [5–8].

Lipases are triacylglycerol ester hydrolases (EC 3.1.1.3) that act on a wide variety of substrates such as triacylglycerides, esters of fatty acids, and lipids from synthetic or natural oils [9–19]. Their natural action involves the hydrolysis of triglycerides to free fatty acids and glycerol. However, acyl transfer reaction on the hydrolysis of ester bonds can also create C–C bonds, acting in a wide range of solvents, making them one of the most widely used enzymes in industrial processes [6,20]. Their main biotechnological applications are in the biotransformation of oils and fats in food, pharmaceutical, cosmetic, and power production industries [21–34].

Lipases can be from animal, microbial, and plant sources, with varying properties [12,21,35]. However, almost 50% of the commercial volume of lipases is produced only from yeasts and filamentous fungi [36,37]. Lipase structure is built on the  $\alpha/\beta$

hydrolase fold composed of a core of eight predominant parallel  $\beta$  filaments, forming a twisted central  $\beta$  sheet, surrounded by a variable number of  $\alpha$  helices [38,39]. Their catalytic triad is composed of nucleophilic serine, histidine, and glutamate or aspartate [38,39]. They also have an oxyanion orifice responsible for stabilizing the oxygen ion formed as an intermediate reaction during catalysis [35,40]. Lipases usually have a chain of hydrophobic amino acids that covers the active site called the lid, which acts to control the access of the substrate to the active site of the lipase, giving it an open–closed shape, which can be conformationally changed by the presence of a polar–nonpolar interface [38,41].

Despite the presented advantages, lipase production suffers from non reproducibility and low yields in their cultivation stage [42,43]. Furthermore, regarding their application, free-state lipases (or free enzymes in general) have limitations such as sensitivity to the reaction medium and low operational stability, making their industrial use practical only by their immobilization [44–46]. Immobilization methods are good alternatives that can favor enzyme activity, facilitate biocatalyst recovery, modulate its selectivity and specificity, and improve resistance to inhibitors [44–59].

Meeting with the current public policies for green and sustainable development, the interest in using immobilized lipases in industrial processes results in increased funding for the studies thereof, causing new support materials and immobilization processes to be discovered and improved [60–68]. In this sense, this study presents the latest research trends in the production of lipase biocatalysts and their optimized industrial applications.

## 2. Novel Techniques for Lipase Immobilization

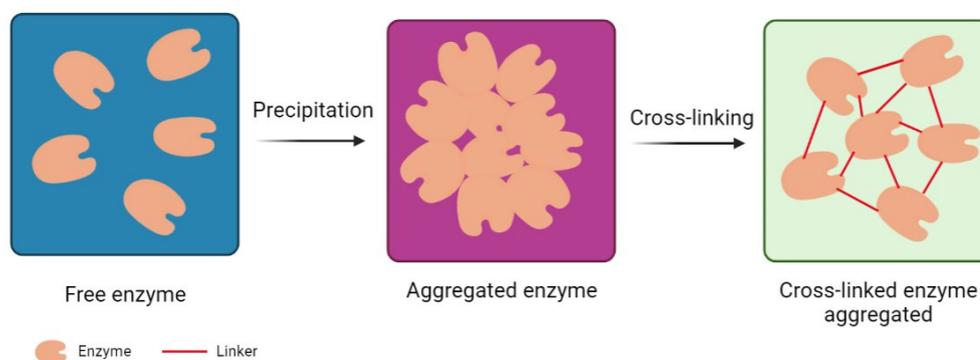
Physical and chemical interactions between supports and enzymes define the linkage of the immobilized biocatalyst [69–73]. The developed technologies and methods for this procedure still use one or more basic strategies: adsorption, encapsulation, covalent bonding, entrapment, and crosslinking. Thus, they may retain their base method's good and bad qualities [6,69,74–76].

Adsorption is the simplest method, which requires the use of reactants [77]. The physical and chemical groups of the matrix interact with the enzyme and retain it [78]. In this way, this method is more susceptible to enzyme leakage [77,78]. Encapsulation is a method that maintains the enzyme in its original conformation and brings excellent mechanical and storage stability [79]. On the other hand, the substrate may have difficulty accessing the enzyme's active site, leading to mass transfer limitations and reduced yields of reactions [79,80]. Entrapment is a similar technique, but it uses a different matrix type, is more accessible, cheaper, and allows for a better substrate diffusion [78]. However, it generally has low operational stability and higher enzyme leakage [78,81,82]. Covalent bonding is the most used method, which requires chemical reactants to create functional groups that will bond with the enzyme [6,69,83–87]. This strategy can offer significant operational stability and reusability to the biocatalyst, but it may cause conformational changes in the enzymes during the immobilization procedure, reducing or even extinguishing enzyme activity [6,74,88]. Using the principles briefly presented in this paragraph, the following subsections present the trending protocols for lipase immobilization.

### 2.1. Crosslinked Enzyme Aggregates (CLEAs)

The technique of immobilization by enzymatic aggregates (CLEAs) is characterized as a helpful and straightforward technique, having such advantages as permanent insolubility and excellent thermal stability [89,90]. Using this method, the structure of the enzyme is conserved, retaining the activity of its catalyst. Furthermore, it allows two or more enzymes to co-immobilize more quickly than other immobilization protocols [91,92]. Crosslinked enzymatic aggregates are among the recent immobilization methods that do not use solid supports and have been requested over the past few years for their ease and robustness, in addition to promoting high specific activity and not requiring highly purified enzymes [93,94]. It is a protocol that combines different preparation steps, such as purification, precipitation, and immobilization, in one, making the process simpler and

faster [95,96]. Thus, they are prepared by enzymatic precipitation to form aggregates, produced by mixing the protein with precipitating agents (e.g., ammonium sulfate, organic solvents, or polymers) in aqueous solutions, as shown in Figure 1 [97,98].



**Figure 1.** Representation of precipitation and crosslinking steps for the formation of crosslinked enzymatic aggregates.

Thus, these enzyme aggregates are crosslinked with a crosslinking agent, usually glutaraldehyde [99–102]. The crosslinking step generates the covalent bonds between the amino acids (specific lysines) of the protein molecules, producing an insoluble biocatalyst with high stability and activity [103]. Different concentrations of glutaraldehyde can influence the leaching process of the immobilized enzyme. Low concentrations of glutaraldehyde make the enzyme fixation process easier [104–106]. In contrast, a higher glutaraldehyde concentration enables many aldehyde groups that interact with the enzyme, making the enzyme immobilization process more efficient and preventing leaching [107–109]. The presence of glutaraldehyde as a functional binding agent contributes to high stability in immobilized enzymes. Covalent bonds formed by enzyme amino groups and aldehyde groups decrease molecular flexibility. This fact suggests positively the increased conformational stability of proteins and, consequently, excellent thermal stability [90,110–112].

The manufacturing process of CLEAs is cost-effective as it does not require the use of preexisting expensive carriers and generally affects heterogeneous biocatalysts with high volumetric activities [113,114]. Furthermore, coaggregation agents (co-feeders) such as polymers or proteins are suggested to enhance inter-enzyme crosslinking [115]. It has been successfully applied in the immobilization of several different types of enzymes such as hydrolases [90], oxidoreductases [116], lyases [117], transferases [118], and isomerases [119] used in the manufacturing of chemical products, being subject to an increasing number of analyses [120,121].

In the study by Rehman et al. (2016) [90], a lipase from *Penicillium notatum* (PNL) was immobilized through crosslinked enzyme aggregates using glutaraldehyde (GLA) and ethylene glycol-bis (succinic acid *N*-hydroxysuccinimide) (EG-NHS) as crosslinking agents. EG-NHS aggregates showed higher hydrolytic activities and esterification compared to GLA aggregates. The highest enzyme activity of CLEAs was reached at pH 9.0 and a temperature of 42 °C. Furthermore, there was a significant improvement in thermal resistance after immobilization. Finally, after ten reuse cycles in aqueous media, GLA and EG-NHS crosslinked lipase CLEAs preserved 63.62% and 70.9% of their original activities, respectively, suggesting that this new CLEA lipase demonstrates potential in many industrial applications [90].

In another study by Jin et al. (2019) [92], the lipase r27RCL from *Rhizopus chinensis* was immobilized in octyl-modified microcellular foams (MCFs-C8) using the crosslinking method. As a result, the crosslinked enzymatic lipase aggregates (CLEAs) exhibited better esterification activity than their adsorbed form. Furthermore, the biocatalyst exhibited excellent thermal and mechanical stability and could maintain 69% of the initial activity after five reaction cycles [92]. More recent work by Muley et al. in 2021 [122] was based on the preparation of crosslinked enzymatic aggregates (CLEAs) of lipase from *Aspergillus*

*niger* using fractional precipitation with ammonium sulfate and crosslinking with glutaraldehyde. In the end, lipase CLEAs showed better thermostability than its free form, in addition to retaining more than 65% of its activity for up to four cycles, exhibiting good storage stability for 12 days when stored at  $4 \pm 2$  °C. Furthermore, they were successful in the application for epoxidation of lemongrass oil [122].

## 2.2. Covalent Organic Frameworks (COFs)

COFs are characterized as porous organic polymeric materials, generally crystalline, obtained through polymerizing building blocks of organic binders [123–126]. They have atomically precise porous structures and can have excellent chemical stability in organic solvents, resisting adverse conditions such as acidic and basic conditions to maintain orderly structures and crystallinity [127,128]. In addition, their high stability results from the purely covalent bond and metal-free structures [127,129]. Due to their excellent properties, such as high thermal and chemical stability, large surface area, excellent pore properties, traceable physical and chemical properties, and ease of operation, COFs exhibit excellent performance in the areas of gas storage and separation, analytical chemistry, catalysis, electrical and storage devices, optoelectronics and drug detection [130–133]. Sustainability is one of their most important properties, distinguishing them from other adsorbents; very stable at 250 °C and 450 °C in an inert atmosphere [134].

Generally, these COFs are synthesized through a solvothermal method under adverse conditions, such as high temperature, ranging between 80 °C and 120 °C, high pressure, where the reaction is carried out in a Teflon-lined stainless steel autoclave or a sealed Pyrex tube, and strict deoxidation, where oxygen is removed by freeze-thaw cycling [135–137]. Due to intermolecular interactions, when monomer molecules contain more groups, material synthesis can be affected, making it challenging to form regular crystalline materials this way [138]. Thus, another advantage of COFs is that their organic structure can be modified to adjust their properties, favoring synthesis [139,140].

In recent years, the application of COFs as supports for enzyme immobilization has opened new horizons for researchers due to their excellent and unique properties, such as marked stability, porous and crystalline structure, in addition to a large accessible surface area [141,142]. The integration of enzymes and COFs can occur through different possibilities, including physical adsorption and covalent bonding directly between them or through a binding molecule [143]. Figure 2 shows how this process takes place.

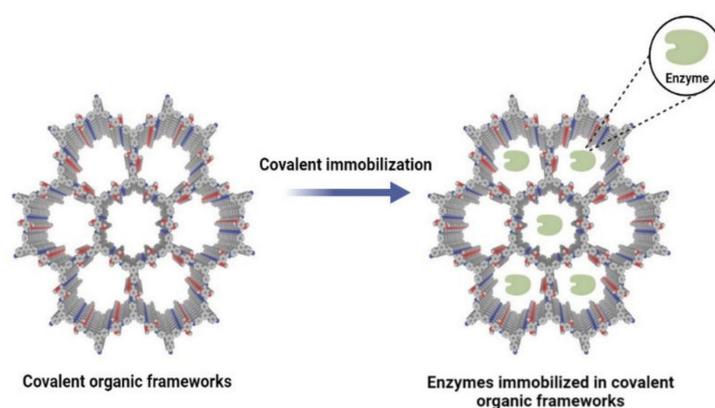


Figure 2. Covalent immobilization of enzymes in COFs.

Catalysts based on COFs have particular advantages over traditional catalysts. Their structures and compositions projectable by chemical crosslinking provide a better understanding regarding the activity and investigation of the catalytic mechanism. In contrast, the highly uniform and adjustable pore structures and sizes facilitate the mass transfer, screening, confinement, and access to catalytic sites [144,145]. The high thermal and chemi-

cal stabilities of COFs ensure that they continue catalytic reaction in various media, which is problematic for some MOFs and inorganic zeolites [144,146].

Currently, there is still little research related to the immobilization of enzymes in COFs. However, studies have been carried out with promising results. According to Zhou et al. (2021) [147], accessible synthesis of a core-shell magnetic COF composite ( $\text{Fe}_3\text{O}_4 @ \text{COF-OMe}$ ) immobilizing *Rhizomucor miehei* lipase (RML) to allow for its application in biodiesel production was reported. As a result, the magnetic structure of COF-OMe achieves highly efficient immobilization and recovery processes and maintains lipase activity to a large extent. The new biocatalyst performed well in practical applications, while the free lipase did not. Furthermore, it successfully produced biodiesel from *Jatropha curcas* oil with a yield of around 70% under optimized conditions [147].

Finally, the process of enzyme immobilization in COF materials is facilitated by their porous structure, high stability, ability to modulate, crystalline, flexible surface area, and the presence of different functional groups [148–152]. These functional groups enable different interactions via hydrogen bonding, hence their increased molecule adsorption capacity. In this context, the immobilization of enzymes in COFs via physical adsorption has an advantage over covalent immobilization. Printing COFs with immobilized enzyme molecules is possible for producing versatile and selective materials [145,153–157] in addition to the possibility of multi-enzyme and porous systems for industrial application.

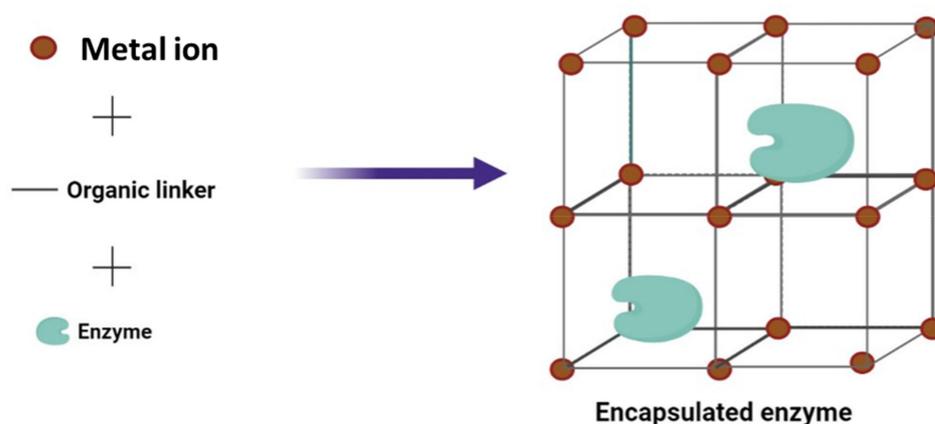
### 2.3. Metal-Organic Frameworks (MOFs)

Metal-organic frameworks (MOFs), also known as porous coordination polymers (PCPs), are built with inorganic nodes and possibly metal ions or clusters with organic ligands [158–160]. This category of materials appears as a promising class with several unique properties such as high porosity, diverse composition, adjustable pore structure, and versatile functionality, bringing merit to different applications ranging from catalysis to storage, separation, purification, and water remediation [161–169].

Opposing traditional inorganic materials, MOFs allow controlling their composition, morphology, pore properties, and function through the careful selection of construction units in addition to the incorporation of intelligent functionalities, expanding and improving their efficiency in specific applications [170–172]. The catalytic activity of MOFs comes from uncoordinated metal centers or functional groups attached to the structure's ligands [173]. In addition, catalysts such as nanoparticles, metal complexes, or biomolecules can be added inside the MOF cage or anchored to its surface as it offers stability to active catalysts and can act as size-selective catalyst support [174–177].

MOFs with the largest surface area and adjustable porosity properties provide the loading of more enzymes than conventional carrier materials. In addition, the shielding effect of their structure allows for stabilization of the enzyme's conformational structure, improving its stability [178,179]. Generally, the preparation of enzymes immobilized in MOFs is based on three strategies: encapsulation, surface immobilization, and pore trapping with presynthesized MOFs [180]. Enzymes encapsulated in porous materials such as porous nanoparticles or reversible micelles have better stability under adverse conditions such as high temperature, organic solvents, or extreme pH [181–183]. Figure 3 presents enzyme encapsulation in MOFs.

In work carried out by Cui et al. (2018) [184], a new MOF–enzyme compound was produced with high stability and easily reusable features through the encapsulation of catalase and ZIF-8 nanocrystals in large layers of mesoporous silica. The obtained system exhibited high activity recovery reaching 81%, and the silica provided a shield to protect the enzyme from biological and chemical degradation. It exhibited excellent stability against proteolytic agents and extreme conditions, such as low pH, in addition to the remaining 50% of the original activity after ten cycles [184].



**Figure 3.** Enzyme encapsulation in metal-organic frameworks.

The work carried out by Li et al. in 2020 [185] was based on the immobilization of the thermophilic lipase QLM from *Alcaligenes* sp. in MOFs through biomimetic mineralization using zinc acetate and adenine as a metal ion and an organic binder, respectively. The immobilized enzyme was successful when applied in the preparation of biodiesel through the transesterification of sunflower oil with methanol, obtaining a conversion more significant than 60% at a high oil/methanol ratio of 8:1. It also showed excellent recyclability during biodiesel production, where no changes in morphology and crystal structure were observed after three cycles. The results proved that the lipase immobilized in bio-based MOFs provided an economical, environmentally friendly, and viable solution for biodiesel synthesis [185].

In the context of enzyme immobilization, MOFs stand out for their high specific surface area, pore-volume, adjustable porosity, high thermal and chemical stability, as well as adjustable mechanical stability [186–189]. MOFs modulate the properties of enzymes. That is, they enable different functionalization, sizes, morphology, and different electrostatic potentials [186–188]. These characteristics are essential to provide excellent stabilization and activity of enzymes at high temperatures, in the media with high acidity or high alkalinity, or in the presence of organic solvents [190,191]. The possibility of synergistic catalysis of MOFs and enzymes is very promising for industrial application [192].

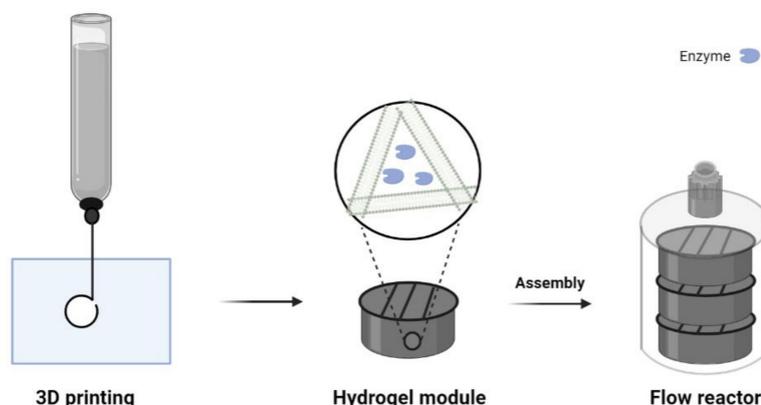
#### 2.4. 3D Printing

In recent years, three-dimensional (3D) printing, also known as additive manufacturing, has emerged as a technology that uses computer-aided design (CAD) for layer-on-layer fabrication, having many advantages over traditional technologies [193–195]. Low cost and endless design possibilities have made this approach very interesting for prototyping in many fields, including process design, aerospace engineering, biomedicine, and catalysis [196,197]. In addition, complex structures with a resolution of up to 0.01 mm can be quickly produced with various materials ranging from polymers to metals [198].

In order to increase compatibility with biological materials, a variety of methods and materials for extrusion-based 3D bioprinting can generate custom hydrogel structures, as shown in Figure 4 [199,200]. These hydrogels can trap enzymes without specific adaptations, offering protection against organic solvents and modulating the reaction rate through mass transfer limitations since 3D-printed structures are relatively thick [201,202]. In addition, chemical modification of printed materials has attracted increasing attention, as by modifying printed carriers, enzymes can be immobilized on them [203].

Enzyme trapping is often applied together with 3D-printed support materials, and, in addition, trapping in this physical material allows the enzyme to be retained in the reactor and provides sufficient purification. The applicability of 3D-printed carriers has been investigated using different enzymes and under different conditions, indicating the universal performance of these materials and their processes [8,204,205]. Therefore, this

new immobilization strategy offers an encouraging possibility to extend the useful life of enzymes [206].



**Figure 4.** Diagram of the 3D printing process for enzyme immobilization.

The study by Santos et al. (2021) [207] produced network-shaped geopolymers successfully prepared by direct ink writing (an additive manufacturing process) to act as carriers for the immobilization of *Candida rugosa* lipase (CRL). The biocatalyst was evaluated in the hydrolysis of residual cooking oil (WCO), a preliminary step for producing biodiesel. The surface of the geopolymer was successfully modified to allow for the covalent bond immobilization process of the CRL. The hydrolytic activity reached  $847.7 \pm 9.7$  U/g and remained more significant than 91% after the first reuse. A free fatty acid content of 75% by weight was obtained from the hydrolysis of WCO, affirming the immobilization efficiency and the suitability of network-shaped geopolymers as support for biocatalysts [207].

In another study, Zhang et al. (2021) [206] built an eco-friendly 3D printing macro-scaffold based on reinforced polylactic acid (PLA) and added phenyl groups with different bond lengths and anchoring two types of combined groups for bonding *Burkholderia ambifaria* lipase YCJ01. The results obtained improved the payload, increased the enzyme expression, ensured 137.3% activity recovery, and increased the specific activity. The biocatalyst was applied to the efficient resolution of racemic 1-indanol (267 mM) using a binary solvent system with high stereoselectivity. In the end, it presented good operational stability with repetitive use for nine cycles, being beneficial to obtain a pure product with high enantiomeric value by viable separation without rigorous operation [206].

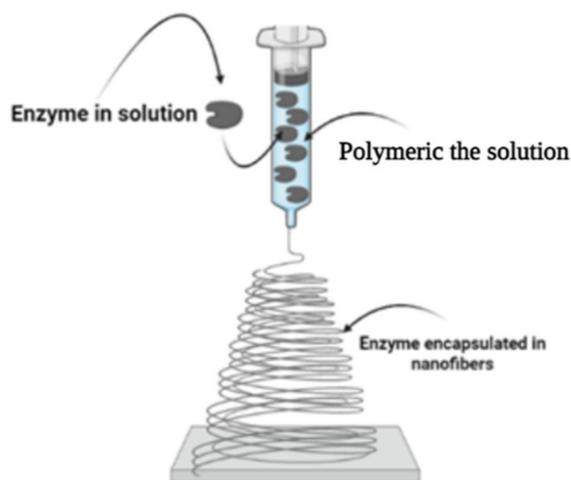
In this way, the immobilization of lipases and other enzymes with 3D structures opens up several possibilities. The possibility of pre-casting these structures with different pore sizes, such as microstructures and porous membranes, is a reality [208–210]. These structures with different groups, receptors, sensitivity to perform immobilization, and production and low-cost materials to achieve these goals make the material produced more competitive for industrial applications [211–215].

### 2.5. Electrospinning

Immobilization techniques have become a rational project subject due to the nature of enzymes and the functionality of immobilization [216–218]. The improvement and innovation in enzyme immobilization techniques made it possible to overcome some common limitations [216–220]. Electrospinning is a practical, simple, and highly efficient technological tool for synthesizing ultrafine fibers with diameters in the range of nanometers [216,219,221,222]. The fundamental principle of this technology is based on the electrostatic forces for the production of nanofibers. High voltage is applied to a polymer solution and the sample collector to produce electric field jets supporting the formation of fibers through solvent evaporation during the process [222,223]. The use of the technique focuses on the main discussions intrinsically connected with the area; it is interpreted as an innovative, effective technique with low cost and versatility that

can be applied in several industrial fields [220,224–226]. The electrified nanofibers have attracted the attention of enzyme engineering and biocatalysis, being considered a potential tool because of their numerous advantages: high surface area, multiple fixation points to the support, high porosity, interconnectivity, high thermal resistance, pH stability, and several solvents [216,220,222,223,227]. The process of immobilizing enzymes in electrified fibers usually promotes the retention and improvement of biological catalytic activity and allows for the easy separation of the enzyme from the proposed reaction environment [216,223,224,228,229].

Note that the surface fixation process refers to the physical adsorption or fixation of enzymes in pure or functionalized nanofibrous supports chemically or physically, and encapsulation means electrospinning of the enzyme and the polymer mixture [219,222,223,228,229]. Electrospun nanofibers have a great potential to overcome dispersion problems, mass transfer limitation, and low recyclability and can be used as suitable supports for the immobilization of several enzymes [216,218,221]. In summary, the notoriety attributed to electrification as an innovative technique is directly connected with the qualification of nanofibers [220,225,227,230]. Aspects such as the diversity of polymers that can be electrophile, high porosity, and chemical interaction of the electrophile support give them a minimal impediment to mass transfer in addition to their industrial usability [216,219,223,225,230]. However, each enzyme interacts differently with the support, so when this technique is chosen, one should pay particular attention to the physical characteristics of the enzyme and the support, always looking at the congruence and adequacy of the size of the enzyme and the pore of the support [216,219,220,223,227,230]. Figure 5 shows how the process of enzyme immobilization by electrospinning occurs.



**Figure 5.** Electrospinning enzyme immobilization method. In this process, the enzyme is dispersed in the solution in which the process of physical/covalent adsorption and encapsulation of the enzyme in nanofibers occurs through the electrospinning process, producing a heterogeneous biocatalyst.

Several studies have reported different proposals to immobilize enzymes in electrophile nanofibers [216,219,223–228]. These proposals mainly include binding the enzyme on the surface of nanofibers and enzyme encapsulation [216,219,223,228].

In this sense, Kuang et al. (2020) [231] reported the development of lipases of *Burkholderia cepacia* (BCL)–SiO<sub>2</sub> (NFM) nanofiber membrane bioreactors prepared through combined electrospinning and enzymatic immobilization strategies. The lipase loading capacity increased drastically, and a certain favoring in the thermal and solvent stability of the biocatalyst formed by electrospinning was noticed, highlighting the technique as efficient for this protocol. Activity remained above 80% after five cycles [231]. In contrast, Isik et al. (2019) [232] synthesized PVA/Zn<sup>2+</sup> nanofibers using electrospinning and then successfully mobilized the lipase into electrospun nanofibers. The results obtained showed the efficiency of the protocol through the improvement of the immobilization parameters of the formed

biocatalyst. Interpreting the immobilization results, the catalytic derivative formed had an increase in the stability properties of the enzyme, such as thermal stability, pH stability, and reusability. Furthermore, it is noteworthy that the immobilized nanoelectrospun biocatalyst protected 90% of the catalytic activity after 14 reuses [232]. This indicates that the recovery of heterogeneous biocatalysts formed using electrospinning protocols is desirable for applications in wastewater treatment industries, drug production, and the field of cosmetic production [231,232].

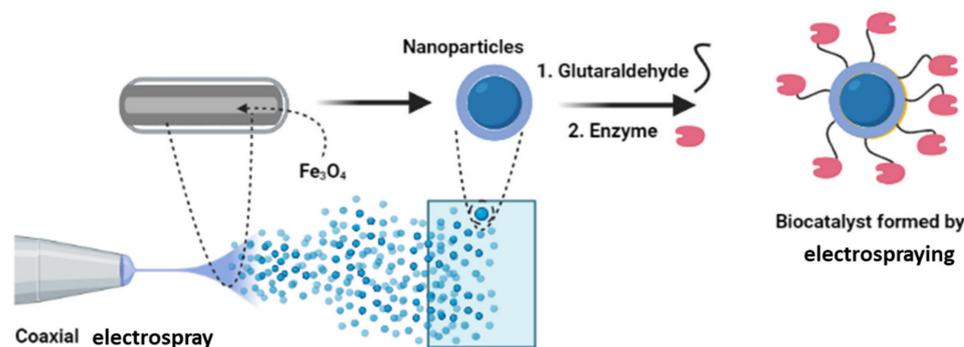
In summary, the immobilization method by electrospinning has proved to be a potential alternative to overcome several daily limitations resulting from the scarcity of natural resources to synthesize new enzyme immobilization matrices. In addition, the versatility of this method provides its wide industrial application.

### 2.6. Electrospraying

The electrospraying technique, popularly known as electrohydrodynamic atomization (EHDA), is a potential technology similar to electrospinning to synthesize polymeric nanoparticles or bioactive fiber-based materials used in various processes [233–235]. Classically, the electrospraying technique is defined as atomizing the liquid through electrical forces [236–239]. The fundamental difference between electrospraying and electrification is the alteration of the properties of the solution, such as solvent concentration, viscosity, and process parameters such as flow rate, distance from the needle tip to the collector, and, mainly, the voltage used [236,237,240]. Moreover, when the concentration of the solution is high, the Taylor cone jet is stabilized, and elongation occurs by the mechanism of whip instability. It is noteworthy that during the process of Taylor cone jet formation in electrospraying, changes in parameters can result in the cleavage of the jet in drops, resulting in the formation of particles of different sizes and shapes [234,236,240]. This process forms micro- and nanoparticles with high loading power and regular particle size distribution [233,234,236,237,239,240].

A few years ago, coaxial electrospraying emerged as an innovative technology for synthesizing products with two miscible or immiscible core and wall materials [233,238,239,241,242]. Coaxial electrospraying is a more convenient one-step method for manufacturing nanoparticles in dry form, thus expanding its industrial applications [233–238]. One of the main advantages of this technique for the immobilization of enzymes is the possibility of designing and adjusting the shape and size of nanoparticles only by modifying the experimental conditions [233,238,239]. This aspect reaffirms the potential of this technique for the enzyme stabilization process in solid matrices [233,237,239,242]. In this sense, electro-pulverized nanoparticles act as a support structure for enzyme immobilization [234,236–238]. A benefit of enzyme crosslinking for particles submitted to electrospraying is excellent residual activity justified by the considerable increase in surface area and porosity [234,236,238,241,243]. In addition, reducing the size of the carrier matter can also improve the efficiency of immobilized enzymes. Thus, the catalytic capacity of enzymes is usually optimized [235,238–240]. However, despite all these advantages mentioned above, there is a scarcity of studies related to this area specifically, to a large degree justified by its only recent discovery [235–238]. Figure 6 below shows the simple process of electrospraying enzyme immobilization.

Liu et al. (2018) [244] immobilized lipases on electrospay fibers with some reinforcement materials such as P(GMA-co-MA)-g-PEO (poly(glycidyl methacrylate-co-methyl acrylate)-g-polyethylene oxide). It is noteworthy that the activity and stability of the derivative were improved, and consequently, the derivative became more versatile, able to be applied in various fields. In summary, the researchers reported that the results showed that the immobilized lipase has good thermal stability, reusability, and stability in organic solvents. The good stabilities of the immobilized lipase revealed that the electrospay fibrous membrane P(GMA-co-MA)-g-PEO is an exceptional carrier for enzyme immobilization [245].



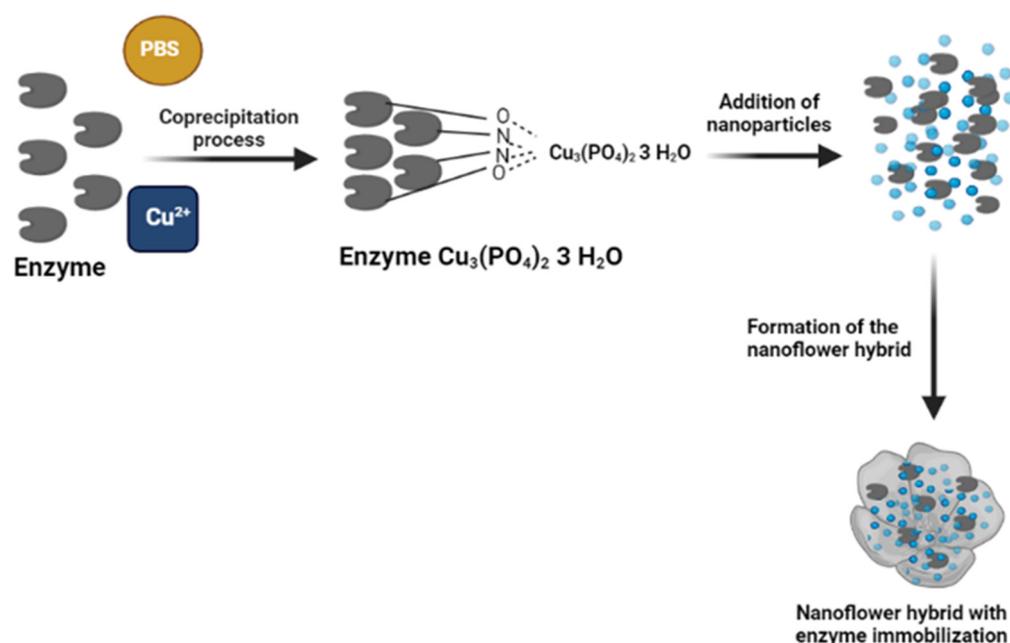
**Figure 6.** Electro spraying enzyme immobilization method. First, iron nanoparticles stop by electro spraying, then they are activated with glutaraldehyde, and then the enzymes bind to the electro spraying nanoparticles by physical/covalent adsorption or encapsulation depending on the size of the enzyme.

In summary, the electro spraying method has become increasingly recurrent in research because it presents advantages different from those commonly highlighted by other classical protocols of enzyme immobilization. In addition, both studies above present the versatility of enzyme immobilization in electroplated supports, perceiving the different areas in which the authors applied their biocatalysts. Despite the great potential of the electro spraying technique for immobilizing lipases and other enzymes, further studies must improve this strategy. It mainly concerns the reproducibility of scale-up methods, economic feasibility, and design of new equipment for the production of nanoparticles by electro spraying, making them competitive for industrial applications [246].

### 2.7. Hybrid Nanoflowers

Hybrid nanoflowers (UFHs) are compounds consisting of organic and inorganic components with a hierarchical three-dimensional nanostructure similar to a flower [247]. Since their inception, these materials have been desirable and desirable for various industry sectors due to their characteristic features such as rapid and eco-friendly synthesis [247–249]. Their high thermal stability, mechanical resistance, and wide surface area constitute their main advantages, enabling these matrices for enzyme immobilization [247,250]. The heterogeneous biocatalysts derived from these preparations are generally veritable and can be applied in several fields: biocatalysis, chemical, and biological analyses, synthesis of chemicals, treatment of pollutants, among many other possibilities [251–253].

It is noteworthy that the increase in the enzyme's thermal stability is usually one of the limiting factors for using free enzymes in high-impact industrial processes [249,254,255]. In addition to this aspect, there is usually an increase in enzyme tolerance by various reactional media, such as organic solvents, and also the primary foundation of immobilization facilitated reuse of the biocatalyst in several reactions cycles [247,248,250]. The synthesis of hybrid nanoflowers is usually straightforward, composed of a reaction of active organic enzymes/molecules and metal ions in aqueous phosphate buffer, usually at pH 7 and 25 °C, generating a hierarchically structured compound with a wide surface-to-volume ratio, retaining a large part of the biomolecules without severe mass transfer limitations [249,250,256]. The preparation typically takes three to five days. However, in a recent study using sonication as an accelerator mechanism, An et al. (2015) [253] reduced this time to just 5 minutes. Many authors used this method to confirm the proposed protocol's efficiency and efficacy [253,254,256–258]. Figure 7 shows how the process for enzyme immobilization by hybrid nanoflowers occurs:



**Figure 7.** Hybrid nanoflowers enzyme immobilization method. First is the coprecipitation of enzymes using PBS and copper ions as an example of an inorganic particle. After coprecipitation, magnetic nanoparticles are usually added to the solution to facilitate separation and reuse. They form an enzymatic biocatalyst of magnetically responsive hybrid nanoflowers.

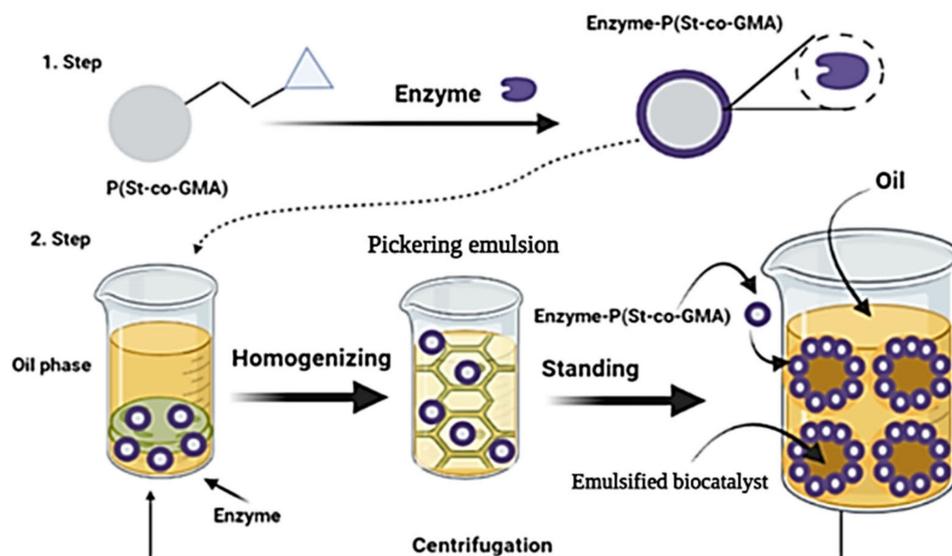
Li et al. (2020) [247] reported the effective production of lipase–inorganic hybrid nanoflowers (NF–lipase) using  $\text{Ca}_3(\text{PO}_4)_2$  as the inorganic component and lipase from *Aspergillus oryzae* as the organic component. The generated biocatalyst was analyzed using physicochemical analyses that confirmed the immobilization of the lipase in the produced nanoflower. Furthermore, the catalytic derivative showed high catalytic activity, maintaining activity above 50% after seven consecutive reaction cycles. Furthermore, it is noteworthy that the HNF–lipase exhibited increased stability against high temperature and denaturants, obtaining good storage stability and reusability [259]. Liu et al. (2020) [260] immobilized the thermophilic lipase QLM from *Alcaligenes* sp. successfully in hybrid inorganic nanoflowers based on  $\text{Cu}_3(\text{PO}_4)_2$  through biomimetic mineralization. The catalytic derivative showed high thermal stability, maintaining catalytic activity above 70% after eight reaction cycles at temperatures between 65 °C and 70 °C [260]. In these examples, the efficiency of this new immobilization strategy is noticeable, primarily when researchers aim mainly to maintain the catalytic activity at high temperatures.

The preparation of biocatalysts via an HNF–lipase requires a better understanding of their molecular interactions. Interactions of ions with lipases must be understood to modulate enzyme activity and increase stability. Thus, the controlled production of these enzymatic biocatalysts would be improved. Furthermore, enabling and reusing these biocatalysts in continuous and efficient processes is a challenge to overcome for industrial applications.

### 2.8. Pickering Emulsion Enzyme Encapsulation

The emulsion process in chemistry is characterized as a mixing of two or more liquids, in which one of them must be present in the form of microscopic droplets dispersed by the other liquid [261–263]. Emulsions are spontaneously synthesized. However, mechanical agitation, ultrasound, and other physical tools are used to accelerate the process [263–265]. It is worth remembering that there are several types of emulsions in chemistry, characterizing this technique as one of the most versatile; it can be applied in many fields such as the food industry, pharmaceuticals, and cosmetics [263,266,267].

Specifically, a Pickering emulsion makes use of solid particles used alone as stabilizers, which accumulate at the interface between two immiscible liquids and stabilize the droplets against coalescence [266–269]. The reactional process of heterogeneous catalysis has gained much attention due to several aspects such as product–particle facilitated separation, minimal particle toxicity, and high catalytic activity process [262,264,268,269]. In addition, current studies show the very high tolerance of this emulsion to organic reactional environments (hexane, methanol, acetonitrile, tetrahydrofuran, among others) [262,263,265,269]. To improve the understanding of the Pickering emulsion enzyme encapsulation method, Figure 8 explains each step in greater detail.



**Figure 8.** Enzyme-decorated nanoparticle-stabilized Pickering gel emulsion for biphasic biocatalysis or Pickering high internal phase emulsions (HIPEs). In step 1, an enzyme-decorated polymeric nanoparticle is prepared to stabilize oil-in-water HIPEs by emulsion copolymerization without a styrene surfactant and glycidyl methacrylate. Then, the second step is adding the Pickering gel system of the enzyme decorated in the nanoparticle. Then, the emulsion is homogenized to increase the interaction of the nanoparticle with the emulsion. Subsequently, standing occurs and finally forms an emulsified biocatalyst using the Pickering method.

In this perspective, the area of enzymatic biocatalysis has been very interested in this process, given the low costs of developing protocols and the high catalytic response offered by the emulsified catalytic derivative [264,269–271]. This vehemence comes from the large “oil–water” interface area that allows biphasic reaction systems to have high efficiency in optimizing hydrogenation, oxidation processes, and enzymatic reactions [269,270,272–275]. Wang et al. (2017) [268] presented a simple strategy for immobilization of the lipase of *Candida* sp. at the oil/water interface of Pickering emulsions via covalent enzyme coupling with CHO-JNPs for organic/aqueous biphasic catalysis [268]. Sun et al. (2020) [267] demonstrated a protocol similar to that of Wang et al. (2017) [268]. However, Sun et al. (2020) [267] performed the ultrasound-assisted process to optimize the immobilization process, in addition to having used the lipase from *Candida rugosa* (CRL) as an enzyme [267]. In this sense, Sun obtained a more active catalytic derivative with 177 mg/g of activity while Wang—only 23.3 U/mL. In addition, Wang’s derivative retained this activity further, maintaining the catalysis power at 88.6% after 10 cycles, while Sun maintained only 75% after nine reactional cycles [267,268]. That said, it is noticeable that ultrasound as an optimization tool in these circumstances is exceptional because it usually allows the enzyme to have more contact with the water/oil interface [263,266,268,269,272,275].

Therefore, Jiang et al. (2020) [276] presented a new Pickering interfacial biological catalysis platform with efficient encapsulation of the lipase of *Candida* sp. composed of binary particles and high catalytic performance. The enzymatic derivative showed

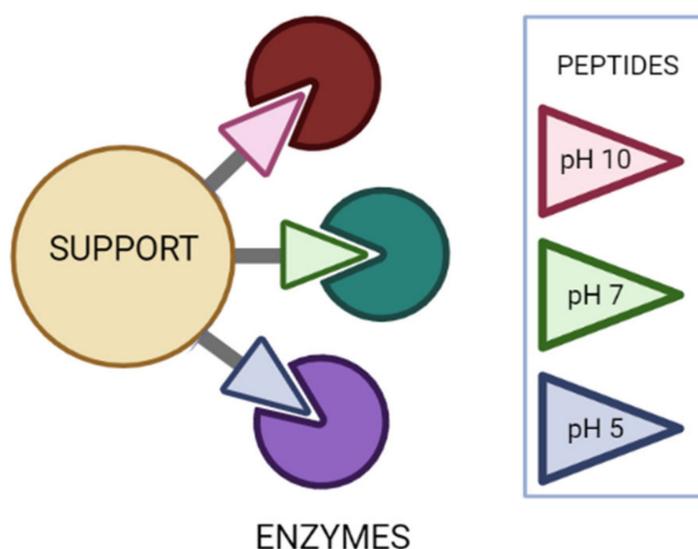
excellent stability, structural integrity, and exceptional reuse [276]. This approach has allowed overcoming one of the main limitations of Pickering emulsions, which is easy leaching. The authors highlight that the use of reinforcement particles is fundamental to improving the interaction of the enzyme with the emulsion interface, offering more significant surface area and greater affinity for the active functional groups of the emulsion with the inactive region of the enzyme [266,268,276].

In summary, this new strategy of enzyme immobilization has gained increasing interest from large industries due to its low-cost processes and numerous additional advantages, prioritizing the latent possibility of its development on the industrial scale almost without operational limitations. The production of enzymatic lipase biocatalysts via Pickering emulsions needs to overcome some challenges. In this context, stability, activity, and durability must be improved. It will be possible to produce them within a large-scale, reproducible, and economically viable industrial application.

### 2.9. Peptide-Guided Immobilization

Enzyme immobilization aims to enhance the catalytic characteristics of these enzymes, and thus different immobilization methodologies are currently being developed [277]. Therefore, peptides are being applied in these processes to increase the efficiency of immobilization. These peptides have hydrophobic characteristics that allow their use in aqueous or polarized media and such media as oils or bio-solvents that do not feature polarization [278]. In addition, peptides can be modified as their side chains to define their hydrophobic and hydrophilic properties more specifically [279]. These modifications in the chains of a peptide guarantee its application on more substrates, regardless of its polarity [280]. In simulations and analysis, it was possible to verify that most peptides tend to present an  $\alpha$ -helical shape in nonaqueous (polarized) environments [281].

Another way to use peptides in enzyme immobilization would be to combine proteins with peptides, thus performing a specific and oriented binding appropriately [282]. This combination can preserve the enzyme's binding to the support and maintain its biological activity. Peptide-guided immobilization was used with the *Escherichia coli* biofilm as support and kept the catalytic properties of the enzymes unchanged [283]. In this case, a differential would be the possibility of undoing the connection when using low pH solutions, thus allowing the use of the materials in other future applications [284]. Figure 9 allows the visualization of peptide-guided immobilization at different pH levels.



**Figure 9.** Peptide-guided bonds where different enzymes are immobilized on the same support that has regions with different pH.

Such applications and characteristics presented by peptides and polypeptides reinforce that its application as a guide for enzymatic immobilization is possible. Furthermore, their properties adapt well to different environments. Therefore, studies with peptides resulted in more application possibilities and more efficient immobilization processes. One of the most promising strategies for lipase immobilization is the technique of using peptides. They can be obtained from different materials and have facilitating characteristics for immobilization systems, such as size and sequences. However, understanding the interaction mechanics between peptides and proteins in the immobilization process is necessary to obtain active and stable enzymatic biocatalysts. Optimizing these systems to make them economically competitive for the industry is another challenge to be overcome.

### 3. Novel Carriers for Immobilization

Several support materials of different origins can be used for enzyme immobilization. Many desirable characteristics influence the choice of enzyme carriers, like the functional groups and charge on the surface, homogeneous particle size, pore size distribution, high surface area, biocompatibility, low cost, inertness towards the enzyme and all the components of the reaction media, microbial resistance, thermal stability, and mechanical stability for continuous production in reactors [79,285–287]. Regarding enzyme immobilization, the carrier's surface functional groups and charges determine its interaction with the enzyme [288–290]. The surface area and porosity of the matrix are the parameters of influence on the amount of the enzyme that can be immobilized [291–293]. Recent research into new materials has contributed to new supports for immobilizing lipases with better features. As shown in Figure 10, these new materials include waste biomaterials, nanomaterials, synthetic resins, mesoporous and electrospun materials. Table 1 also contains detailed information from recent studies [81,294–296].

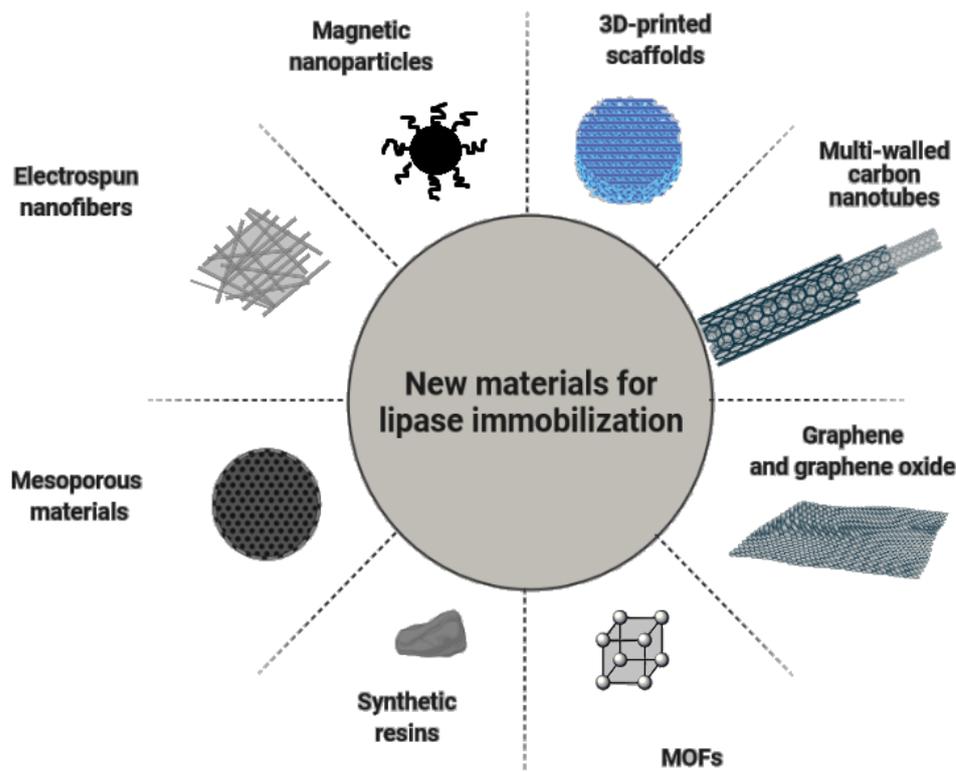


Figure 10. Novel materials used for lipase immobilization.

**Table 1.** Novel research in lipase immobilization.

Support	Lipase Source	Application	Immobilization Technique	Ref.
Virus-like mesoporous organosilica nanoparticles	Lipase B from <i>Candida antarctica</i>	Synthesis of levulinate esters	Covalent bonding	[297]
Lifetech <sup>TM</sup> methacrylic synthetic resins	<i>Thermomyces lanuginosus</i>	Biodiesel synthesis	Physical adsorption	[298]
MIL-101(Cr) MOFs	<i>Candida rugosa</i>	Hydrolysis of <i>p</i> -nitrophenyl palmitate	Covalent bonding	[299]
Chitosan–mesoporous silica hybrid nanomaterials	Porcine pancreatic PPL	Triacetin hydrolysis	Covalent bonding	[300]
Magnetic multiwalled carbon nanotubes	<i>Candida rugosa</i>	Synthesis of fruit flavors	Covalent bonding	[301]
3D-printed carbon fiber-reinforced polylactic acid scaffolds	<i>Burkholderia ambifaria</i>	Hydrolysis of <i>p</i> -nitrophenyl palmitate	Adsorption	[203]
Inorganic hybrid nanosheets on sulfonated macroporous resins	<i>Candida rugosa</i>	Hydrolysis of the olive oil emulsion	Encapsulation	[302]
Nanocellulose-fused polypyrrole/graphene oxide nanocomposites	<i>Candida rugosa</i>	Synthesis of fruit flavors	Adsorption	[303]
Poly(carboxybetaine methacrylate)-grafted silica nanoparticles	<i>Candida rugosa</i>	Hydrolysis of <i>p</i> -nitrophenyl acetate	Covalent bonding	[304]
Spherelike bacterial cellulose	<i>Rhizopus chinensis</i>	Hydrolysis of the olive oil emulsion	Covalent bonding and physical adsorption	[305]
Electrospun nanofibrous membranes containing epoxy groups and a hydrophilic polyethylene oxide chain	Lipase B from <i>Candida antarctica</i>	Hydrolysis of olive oil	Covalent bonding	[244]
Pyrolyzed sugar industry waste	<i>Aspergillus</i> sp. lipase	Synthesis of 2-phenylethyl butanoate	Adsorption	[306]
Octyl Sepharose crosslinked with dextran aldehyde polymers	<i>Thermomyces lanuginosus</i> , <i>Rhizomucor mieheii</i> , and lipase B from <i>Candida antarctica</i>	Hydrolysis of <i>p</i> -nitrophenyl butyrate	Covalent bonding	[307]
Colloidal lignin particles	Lipase M from <i>Mucor javanicus</i>	Synthesis of butyl butyrate	Entrapment	[308]
Chitosan/nanocellulose biocomposites	<i>Candida rugosa</i>	Synthesis of butyl butyrate	Covalent bonding	[309]
Magnetic rice straws	<i>Thermomyces lanuginosus</i>	Synthesis of biodiesel	Covalent bonding	[310]
Diethylenetriamine-modified magnetic cellulose beads	Lipase B from <i>Candida antarctica</i>	Synthesis of biodiesel	Covalent bonding	[311]
Chitosan–chitin nanowhiskers	<i>Rhizomucor mieheii</i>	Synthesis of eugenyl benzoate	Covalent bonding	[312]

Biopolymers can offer low cost, easy modification, high surface area, and various functional groups to stabilize lipase conformation [286,294]. As for drawbacks, it can be mentioned that their poor mechanical stabilities often make them unsuitable for industrial applications in bioreactors [313,314]. Chitosan, a trending biopolymer, also has low chemical and long-term stability [294]. The problems of enzyme leaching and stability of biopolymers have been addressed by using crosslinking agents and by the combinations of biopolymers to produce biocomposites [315,316]. The use of crosslinking agents requires attention to the possible adverse effects on lipase conformation, which can cause loss of activity. Concerning the formulation of biocomposites for lipase carriers, it is necessary to be careful with the produced biocomposites' hydrophilicity since it can also reduce the activity of the biocatalyst [317]. Current research often identifies new crosslinking agents and biocomposites that have properties superior to the existing materials [28,318,319].

Biopolymers and biocomposites are used to produce different types of lipase matrices, conjugated or not, with a broad range of ligands of distinguished properties and nature, as mentioned in recent studies: membranes, resins, nanofibers, beads, nanoparticles, nanotubes, scaffolds, colloidal particles, and nanowhiskers [203,244,298,305,308,309,312]. In the study of Elias et al. (2018) [309], the biocatalyst consisting of a biopolymer carrier of nanocellulose/chitosan was prepared by extraction of nanocellulose from palm oil frond leaves, followed by consecutive crosslinking with chitosan and the *Candida rugosa* lipase,

both using glutaraldehyde as crosslinking agent [309,320]. The prepared biocatalyst was applied in butyl butyrate synthesis, showing good thermal stability, and retained about 50% of its initial activity through seven consecutive recycles [309].

In another study, spatially confined biocatalysts upon self-assembly and drying-driven aggregation of Lipase M from *Mucor javanicus* cationic lignin nanosphere (c-CLP) complexes were prepared in the calcium alginate hydrogel [308]. This innovative procedure showed retained activity superior to the commercially available immobilized biocatalysts in the same conditions in butyl butyrate synthesis [308].

Another possible strategy is to incorporate inorganic materials to produce hybrid biocatalysts with unique properties [297,300,303,310,311]. Similarly, inorganic materials can be conjugated with organic or inorganic substances to produce other hybrid biocatalysts, as mentioned for many kinds of nanomaterials, metal-organic frameworks, mesoporous and ceramic materials [81,295,296,299,301,302,304].

In the work of Jiang et al. (2019) [297], a novel matrix for lipase immobilization was produced [297], hydrophobic virus-like organosilica nanoparticles (VOSNs) with a spherical core formed by mesoporous organosilica surrounded by epitaxial perpendicular nanotubes. They were made using the epitaxial growth method with hexadecyl trimethyl ammonium bromide as a template (CTAB) and tetraethyl orthosilicate (TEOS) and 1,2-bis(triethoxysilyl)ethane (BTSE) as silica sources. In a typical synthesis, CTAB was dispersed in deionized water under stirring. Then, an aqueous sodium hydroxide solution was added to the water solution and stirred for two hours at 60 °C. After that, a solution of TEOS and BTSE in cyclohexane was added dropwise. The reaction was continued by stirring the mixture at 60 °C for forty-eight hours. The product was collected by centrifugation, washed several times, and dispersed in acetone for refluxing treatment at 80 °C for thirty-six hours to remove the CTAB template. After that, the surface was modified by 3-aminopropyl triethoxysilane (APTES) activated with glutaraldehyde and covalently bonded with lipase B from *Candida antarctica*. The prepared biocatalyst exhibited suitable pH, thermal, organic, and storage stability. It was also demonstrated that the prepared biocatalyst exhibited superior reusability compared with free and immobilized lipases in virus-like silica nanoparticles [297].

Zare et al. (2018) [299] studied the effect of different chemical modifications of the MOF chromium terephthalate (MIL-101(Cr)) for immobilization of the *Candida rugosa* lipase [299]. Amino, trichlorotriazine amino, and glutaraldehyde amino were the groups added to the carrier surface, and the last two showed higher pH, thermal, and storage stability, although all the preparations significantly lost activity in the reusability tests. According to the authors, these results were related to the biocatalyst contamination by the substrate, meaning that the biocatalyst could potentially use other types of substrates [299].

For 3D-printed materials, the fused deposition modeling (FDM) technology is based on extrudable thermoplastics, having the advantages of being low-cost and straightforward. In addition, the used fibers are cheap and easy to recycle, therefore reducing the operational costs [321]. In the work of Ye et al. (2019) [203], carbon fiber-reinforced polylactic acid 3D-printed scaffolds of different structural shapes were used to create integrated reactors carrying four types of enzymes including the *Burkholderia ambifaria* lipase, showing great recovery activity [203].

#### 4. Concluding Remarks

Nanotechnology is occupying even more space in biocatalysis. This tool makes it possible to create carriers with specific features to improve operational stability, immobilization, and reaction yields. Still, it is necessary to reduce biocatalyst production costs, which are the main issue for their industrial applications. The use of waste materials is an exciting alternative since they are cheap and meet the growing environmental needs of the market. Other trending research focuses on genetic manipulation of lipases, co-immobilizing multiple lipases on the same matrix, and hybrid materials. Those increase process complexity and costs and can be further employed for the industrial use of immobilized lipases.

As for new immobilization techniques, it is essential to emphasize that the choice of the method, support, and enzyme directly influences the process and is dependent on several factors. The various examples cited throughout the work demonstrate the results obtained by several researchers from the use of different conditions, involving not only these three factors but many others (temperature, pH, reaction time). For the choice of lipase, it is essential to know the factors that influence its activity and the desired characteristics for its final application. At the same time, in choosing the method, it is essential to consider issues such as regeneration and deactivation of the enzyme, the cost of the process, and the toxicity of the reagents.

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