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Review

Recent Developments in Carriers and Non-Aqueous Solvents for Enzyme Immobilization

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Abstract: Immobilization techniques are generally based on reusing enzymes in industrial applications to reduce costs and improve enzyme properties. These techniques have been developing for decades, and many methods for immobilizing enzymes have been designed. To find a better immobilization method, it is necessary to review the recently developed methods and have a clear overview of the advantages and limitations of each method. This review introduces the recently reported immobilization methods and discusses the improvements in enzyme properties by different methods. Among the techniques to improve enzyme properties, metal–organic frameworks, which have diverse structures, abundant organic ligands and metal nodes, offer a promising platform.

Keywords: enzymes; immobilization; adsorption; cross-linking; covalent bond; non-aqueous solvents; metal–organic frameworks; cross-linking; thermostability

1. Introduction

Enzymes are biological macromolecules that are generally globular proteins [1]. They are known to catalyze numerous chemical reactions [2,3] and are widely used in different areas, such as the food, agricultural, cosmetic and pharmaceutical industries [4]. Moreover, compared with reactions that use hazardous chemical solvents, enzymatic reactions have remarkable advantages, such as fewer reaction steps, low cost, sustainability and are environmentally friendly, especially in biodiesel production applications [5]. The reusability or stability of enzymes used in industrial applications are key factors in reducing costs. To achieve this objective, immobilization techniques have been used for decades. In some cases, immobilization can enhance the activity of enzymes, protect enzymes from harsh conditions or stabilize enzymes during reactions [6–8].

Generally, immobilization techniques mainly include adsorption and covalent bonding (Figure 1) [9,10]. The main differences between these techniques are the type of interaction between the enzymes and support materials. In order to pursue a new method of immobilization, a suitable support needs to be found that is mesoporous, hydrophobic/hydrophilic, stable in reaction, and has surface activity.

In recent years, new materials such as magnetic materials, metal–organic frameworks, and new polymers or porous inorganic materials have driven more interest. In this review, we provide an update on studies from the past five years and discuss improvements in enzyme properties and the limitation of the application of immobilization.

2. Immobilization Techniques

2.1. Adsorption

The most widely used immobilization method is adsorption, which is mainly based on van der Waals forces, generated by physical interactions between the solid carrier and enzyme, forming weak bonds [11]. The immobilized enzyme can contact the solid carriers for a fixed time period under suitable conditions. Adsorption is an easy and low-cost process and does not disturb the enzyme structure or active sites during binding to the substrate, helping maintain the enzyme activity. Adsorption is still the simplest and most traditional method of immobilization. In recent studies, there has still been a focus on silica-based supports, magnetic particles, polyvinyl alcohol, polysaccharides and also other commercial products such as ECR8285 and SBA-15 [12–14]. These studies mainly suggested that the adsorption method can improve the substrate selectivity or enhance enzyme stability and reusability. However, due to the weak bonds between the enzyme and carrier, adsorption can be altered by pH, temperature or other factors such as ionic strength, resulting in desorption. In recent years, more studies have investigated supports that have a mesoporous, larger surface and higher affinity to the substrate.

Recently, phyllosilicates such as sepiolite have attracted more interest because of their high specific surface areas, high capacities and silanol groups. Lipases immobilized on montmorillonite exhibited higher catalytic activity than nonimmobilized lipases. In that report, modified and unmodified sepiolite and montmorillonite were tested for immobilization, and the hydrolytic activity was increased two times in the case of organo-modified sepiolite compared to unmodified sepiolite [15].

Synthetic polymers such as diethylaminoethyl cellulose (DEAE cellulose) can adsorb 576 units of peroxidase per gram onto their matrix structure [16]. Some synthetic polymers are immobilized in oppositely charged layers of solid support by different pH buffer washes. These layers can be modified into layers of a suitable thickness and assembled in a multilayer form by cationic/anionic species to adsorb enzymes. Overcoming synthetic challenges, such as architecture control and functional complexity is a key factor in stabilizing the enzyme during a reaction.

Porous polymeric materials, such as liposomes, are prepared as semipermeable membrane coatings [17,18], which play a pivotal role in various applications, such as drug delivery and food science [19,20]. The polyelectrolyte complex structure, which has a charged block or a neutral hydrophilic block, allows enzymes to be encapsulated and applied for drug delivery [21]. Bovine serum albumin encapsulated by a chitosan-carrageenan polyelectrolyte complex was assessed in a process simulating the gastrointestinal tract environment [22–24]. The encapsulation method normally helps enhance catalytic efficiency but is limited to enzyme loading and mass transfer.

Silica materials are known to have mesoporous structures [25], and their narrow pore size distribution and large surface area offer a good environment for contact. In recent studies, the small pores of the silica SBA (Santa Barbara Amorphous)-15 have been increased from approximately 5 to 30 nm in diameter [26], the volume of the mesopores has been reported to be approximately 0.8 cm^3/g, and the surface area has been increased up to 1400 m^2/g [27]. In silica gels, various pore sizes from 70 nm to 250 nm have been obtained [28,29]. Additionally, a non-ordered mesoporous silica material, Hiroshima Mesoporous Material, with a large pore size and no regular shape was prepared in a water/oil phase through organic templates [30,31]. The size differences among silica materials offer many possibilities. Metal oxides have also been applied in many areas, such as ferrous titanium and aluminum oxides [32–37].

In summary, the adsorption method possesses high enzyme loading ability, has minimal effect on enzyme conformation, and can maintain enzyme activity; however, the weak physical bond is sensitive to temperature, pH and other factors. Reusability is also still an issue for the application of the adsorption method.

2.2. Covalent Bonding

In covalent bonding, which is the most important immobilization technique, enzymes form covalent bonds with the support material through side-chain functional groups, such as carboxyl groups and amino groups [38,39]. The functional groups are present in the side chains of amino acids, mainly including lysine, arginine, aspartic acid, and histidine. These functional groups are not responsible for the catalytic activity, so the activity of the enzyme can be retained. The enzyme linkage enhances enzyme activity, stability, and thermostability and prolongs enzyme half-life [40–43]. Enzymes conjugated onto polymers by site-specific immobilization through reaction of thiol groups from cysteine residues with unsaturated carbonyls from the polymer support are widely used [43].

Numerous covalent bond-based methods have been directly used in nanomaterials recently [44,45]. These nanomaterials can be tailored to the target enzyme, and their large surface area and selectivity of porosity provide great advantages in industrial and pharmaceutical products [6,46–48]. For example, immobilization of urease on epichlorohydrin cross-linked carboxymethyl cellulose beads with polyacrylamide resulted in both the optimum pH and temperature shifting higher to 8 and 45 °C, respectively, and enabled 88% of the enzyme activity to be maintained after 10 cycles, and the modified support helped improve the stability of the urease structure [49]. In another case, pectinase was immobilized on chitosan magnetic nanoparticles (CMNPs) through a linker, dextran polyaldehyde. The immobilized pectinase can maintain the initial activity level of the free enzyme and can double the stability in the range of 55–75 °C. In recent studies, naturally derived polymers, specifically cellulose, chitosan, alginate and agarose-based biomaterials have been widely modified and developed. We have listed several examples that have been used frequently in recent years in Table 1, such as magnetic cellulose nanocrystals, commercial ECR8285, which is used for lipase immobilization, and MANAE agarose, which significantly improves enzyme stability and enantioselectivity [13,50,51]. For one enzyme, arymalonate decarboxylase, immobilization on an amino c2 arylate carrier improved the stability of the free enzyme's half-life from 1.2 h to 8.6 days. The covalent bond helps strengthen the structure [52]. Overall, these supports can provide natural microenvironments for enzymes and retain and even enhance their activity while increasing their stability during storage and processing. However, the challenge of retaining the enzyme activity needs to be considered in the process of immobilization [9].

A carrier-free immobilization method is cross-linking, which is based on the formation of cross-links between enzymes. There are two ways to achieve cross-linking enzyme immobilization: a cross-linking enzyme aggregate (CLEA), and cross-linking enzyme crystal (CLEC). Generally, these two methods require cross-linkers, i.e., glutaraldehyde. The cross-linker mainly reacts with lysine residues and forms intermolecular cross-links between two neighboring enzymes [53]. CLEA is a fast, mild and economical method, and its operation normally combines purification and immobilization as a single step [53,54]. The CLEA method can improve enzyme stability, thermostability, and activity, and can protect enzymes from organic solvents or autoproteolysis [55,56]. In addition, mesoporous silica gel also provides a high capacity and wide surface area, for example, cross-linking α-chymotrypsin into hierarchically ordered mesoporous silica [57]. CLEC is another cross-linking method; the enzyme needs to be crystallized in a suitable buffer, treated with a cross-linking agent and applied in non-aqueous solvents. CLEC also helps stabilize the enzyme under harsh conditions due to its high stability at different pH levels, in organic solvents and during proteolysis [58]. Lipase immobilized by this method has recently been used in digestion under gastric conditions [59]. We have reviewed and discussed this approach in detail in another paper [60].

In comparison to the adsorption method, covalent bonds help the enzyme attach to the support materials or strengthen itself; however, it may also distort the conformation of the enzyme and further alter its activity. The immobilization efficiency is generally low, and chemical bonding may also denature the enzyme.

2.3. Metal–Organic Frameworks

Metal–organic frameworks (MOFs) are porous crystalline organic–inorganic hybrid materials [61] that consist of coordination bonds between metal ions and organic linkers (Figure 1) [62]. Recently, MOF materials have been well developed due to their ultrahigh porosity (e.g., Cu-MOF (1.78 nm), Tb-TATB (3.9 and 4.7 nm), Mn-MOF (3.4 nm), and PCN-333 (4.2 and 5.5 nm)), diverse functionalities and tremendous surface properties (specific surface areas up to 6000 m^2/g) [63]. They have been used in drug delivery, bioimaging, molecular sensing, gas and molecular separation, etc. [64–67]. Therefore, enzymes immobilized on MOFs have drawn increasing attention recently, and MOF materials such as zeolitic imidazolate framework-8 (ZIF-8), Tb-BDC, MIL-88, and HKUST-1 can maintain enzyme activity well [64,68]; for example, *Candida antarctica* lipase immobilized in B-IRMOFs has up to 1000-fold enhanced activity [69], and the activity of Cyt *c* embedded in ZIF-8 has been increased 10-fold [70].

Enzyme immobilization on MOFs is done using three types of methods: the first is MOF surface immobilization, the second is enzyme diffusion into MOFs, and the last is in situ encapsulation. Therefore, MOFs combine adsorption and covalent bond methods [71].

The first method involves a novel hybrid material that combines silica nanoflowers, ZIF-8 and penicillin G acylase (PGA), showing that PGA integrated with silica nanoflowers and ZIF-8 has better mechanical and chemical stability than PGA integrated with silica nanoflowers only [72].

In the second method, microperoxidase was diffused into a mesoporous Tb-TATB MOF, which provides an environment that enhances the diffusion rate and further improves and retains the enzyme activity after multiple cycles [73]. In a recent report, a new material, NU-1003, immobilized organophosphorus acid anhydrolase (OPAA) and significantly increased the catalytic efficiency of the enzyme [74].

The third method is mainly based on the simultaneous synthesis of a nanomaterial or MOF from an enzyme, an organic linker and a metal ion. Anionic clay compounds in layered double hydroxides (LDHs) have been widely studied recently, for example, flowerlike MgAl-LDH applied in the LDH phase of both monometallic (e.g., ZIF-8, ZIF-67 and Cu-BTC) and bimetallic (e.g., CoZn-ZIF) MOFs. This study showed that MgAl-LDH/ZIF-8 and MgAl-LDH/Cu have good activity, selectivity and recyclability [75].

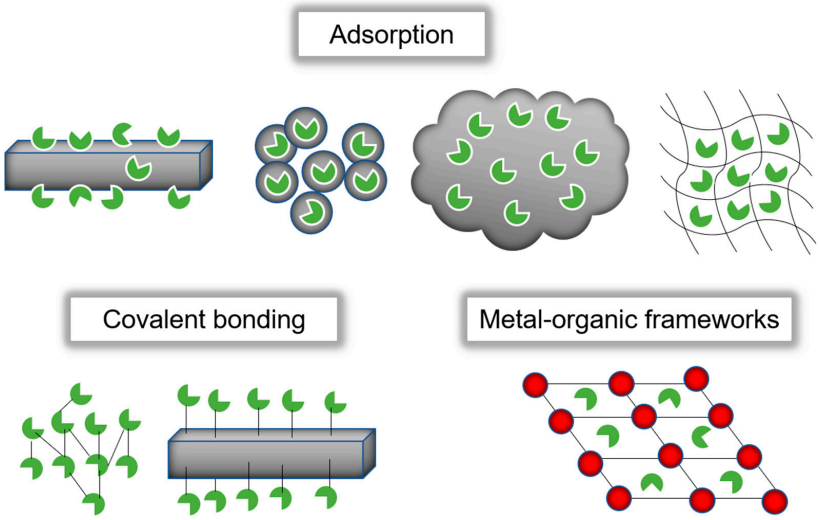

Figure 1. Schematic of various carriers for immobilization of enzymes.

2.4. Non-Aqueous Solvents

Enzyme catalysis in non-aqueous solvents refers to catalytic reactions performed in solvents other than water. In some cases, using enzymes in non-aqueous solvents helps increase their stability [76,77], improve their substrate specificity, stereoselectivity and recoverability, and, in some cases, even increase

their solubility [78,79]. Enzyme-substrate reactions can be significantly improved through this method, especially in cases of low solubility. The most frequently reported example is lipase, where hydrophobic solvents can increase its productivity [80]. Generally, there are two types of non-aqueous solvents: ionic liquids (ILs) [81] and supercritical fluids (SCFs) [82,83] (Figure 2).

2.4.1. Enzyme Immobilization in Ionic Liquids

Biocatalysis in ionic liquids (ILs) [81] was first proposed in 2000 [84,85]. ILs generally contain organic cations and inorganic anions, and IL immobilization can improve the catalytic activity, stability, thermostability and enantioselectivity of enzymes [86–88]. The IL method is often combined with other methods to satisfy the requirements of an application and to help further improve the properties of the enzyme, such as stability, activity and productivity (Table 1). Generally, enzyme immobilization in ILs includes two kinds of methods; one involves the enzyme being encapsulated in ILs and another method involves ILs being modified in various supports. Apart from the traditional supports such as polysaccharides, synthetic resins and polymer networks to encapsulate the enzymes, the novel immobilization on IL-based polymer frameworks has sparked an interest in overcoming decreasing enzyme activity and increasing leaching behavior in the traditional encapsulation method. For example, lipase was mixed with [PPmim][PF_6] at temperatures above 53 °C, and when the mixture was cooled, the lipase was coated with [PPmim][PF_6] in the solid state [89], which helped improve enantioselectivity. CalB was encapsulated in poly(VEIm-Br) and assessed in different solvents. The results suggested that activity was enhanced and there was no leaching of enzyme activity [90]. Enzymatic biodiesel production has been hindered by reusability, but the enzyme-IL system helps reuse of enzymes over more cycles [91]. Another method involves IL-modified supports used to immobilize enzymes, such as IL-based periodic mesoporous organosilica. A recent report used treated surfaces of xerogel silica to immobilize *Burkholderia cepacia* lipase through covalent bonds. The addition of a protic IL during the synthesis of the xerogel silica support significantly helped increase the recovery yield, temperature and pH stability, kinetic parameters and conversion efficiency [92]. Enzymes such as lipase and α-amylase immobilized on liquid-based periodic mesoporous organosilicas and cellulase immobilized on magnetic nanomaterials, which was modified by ILs, were studied. The results suggested that the operational stabilities were all enhanced [93–95].

2.4.2. Enzyme Immobilization in Supercritical Fluids

Among the different types of enzymes, lipases have been the most widely studied in SCFs, as many substrates of lipases are hydrophobic [96]. Supercritical carbon dioxide ($scCO_2$) can provide an excellent environment for the dissolution of hydrophobic substrates, and CO_2 is nontoxic, non-flammable and has low critical parameters [97]. However, in this system, water concentration, temperature and pressure are key factors in the reaction, and these factors can impact the enzyme activity [98]. In recent studies, SCF-based high-pressure packed bed enzymatic reactors or stirred tank reactors combined with various immobilization techniques have been used in industry. For example, the lipase from *C. antarctica* has been immobilized on a ceramic membrane for ester synthesis [99]. In the modern biodiesel industry, reactions of waste animal fat have been catalyzed by the lipase from *C. antarctica* immobilized on methacrylic resin, which increased the yield of the fatty acid methyl ester (FAME) product [100]. In some cases, $scCO_2$ combined with enzymes can help extract more organic product in a clean way; for example, kemzyme can help liberate more polyphenols. The horseradish peroxidase on surface modified mesoporous activated carbon combined with $scCO_2$ enhances the diffusivity and helps remove the phenol in hydrogen peroxide [101,102]. Lipase has been used by this method to increase the conversion rate in many studies, such as *C. Antarctica* lipase making the conversion rate up to 1.16 times higher [103]. In addition, enzymes immobilized into MOF supports combined with $scCO_2$ methods can also obtain higher conversion rates. For example, carbonic anhydrase immobilized into ZIF-8 increases the conversation rate 22-folds compared to free enzyme [68]. In most cases, to enhance

the solubility of a hydrophobic substrate, the enzyme was immobilized on a support carrier in $scCO_2$, as shown by the list of SCF-based examples in Table 1.

In most cases, the stability and activity of enzymes are still the major barrier to their use in non-aqueous solvents. Overcoming these issues will become the key factor in the industrial applications of enzymes.

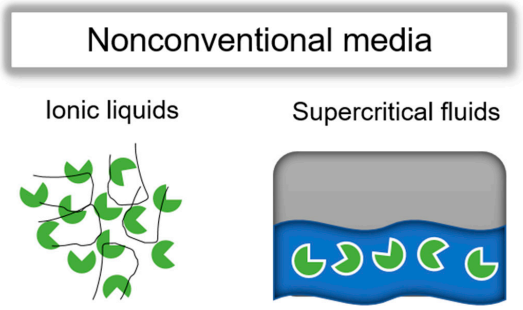

Figure 2. Various non-conventional media and non-aqueous solvents for the immobilization of enzymes.

3. Summary

Immobilization of enzymes on the surface of a material can enhance their activity, and some studies using nanoparticles or nanomaterials, such as graphene, nanotubes and polymeric nanomaterials, as support materials, have reported that enzyme catalytic efficiency was retained or even enhanced [104,105].

Generally, compared to free enzymes, enzymes immobilized by covalent bonds exhibit an improved rigid structure, reduced conformation changes under harsh conditions, and higher activity [106].

The first consideration for improving enzyme stability is the structure of the enzyme itself, although the stability also depends on the properties of the support material, such as the type of interaction, bonding position, enzyme flexibility and microenvironment in the matrix. There is no best method to stabilize an enzyme during immobilization, as the optimal method depends on various factors.

For substrate selectivity, immobilization alters the conformation of the enzyme, further improving the substrate selectivity of the enzyme (Table 1). The microenvironment, such as pore size, can also help improve the substrate selectivity [107]. By using an SCF or a multiphase method, a previously insoluble substrate can be dissolved, improving the substrate selectivity [107].

Among these new techniques, MOFs have drawn considerable attention from many researchers due to their exceptionally high surface area, porosity, abundant metal nodes and diverse structures. The porous structure can prevent the enzymes from forming aggregates and can promote substrate diffusion, further improving the activity. Covalent bonding techniques generally cause changes in the enzyme conformation due to the strong linkage with the enzyme. Encapsulation techniques are easy and rapid methods that have been widely used, but they are limited by leakage, pore diffusion and the limited number of successful industrial applications. None of the conventional medium-based immobilization techniques help improve the solubility of the substrate, but those based on non-aqueous solvents are still limited due to their stability. In recently developed MOFs, such as the expanding csq-net Zr-based MOF [108], the MOF helps improve enzyme localization and the rates of substrate and product diffusion. In particular, the MOF can improve the accessibility of the enzyme and coenzyme. A recent novel and highly efficient hierarchical channel enzymatic system provided a size-dependent channel and windows that can enhance the diffusion rate of the substrate and product, accelerate the kinetic reaction rates, reduce enzyme aggregation and eliminate enzyme leaching. Therefore, the reaction rate was significantly increased. Other solid supports can also stabilize enzymes in variable pH values, and increase their reusability and adjustability, but they will essentially cause distortion of the structure of the enzyme. Therefore, the channel of a MOF provides a better solution. Studies on the expanding csq-net Zr-based MOF have further proven that the advantage of MOFs as a support is their ability to change the enzyme properties to match industrial needs.

Table 1. Typical research on improving enzyme properties by immobilization techniques.

Method	Support Material	Enzyme	Improved Enzyme Properties	Ref
Adsorption	Octadecyl Sepabeads and octyl sepharose resins	*Fusarium verticillioides* lipases	Substrate selectivity	[12]
Adsorption	Accurel MP1000	lipG9	Substrate selectivity	[109]
Adsorption	Modified pullulan polysaccharide	*Burkholderia cepacia* lipase	Substrate selectivity	[110]
Adsorption	SiO_2	*Candida antarctica* B lipase	Substrate selectivity	[111]
Adsorption	Mesoporous silica	Esterase	Substrate selectivity	[112]
Adsorption	TiO_2–lignin hybrid	*Aspergillus niger* cellulase	Thermal and chemical stability	[113]
Adsorption	Mesoporous silica SBA-15	Myoglobin and lysozyme	Acid stability and activity	[14]
Adsorption	Dopamine-functionalized mesoporous onion-like silica	*Candida sp*. 99-125 lipase	Durability	[114]
Adsorption	Epichlorohydrin cross-linked Carboxymethyl cellulose beads	Urease	Acid stability and thermostability	[49]
Adsorption	Polyvinyl alcohol hydrogel	*Xanthophyllomyces dendrorhous* β-fructofuranosidase	Thermostability	[115]
Adsorption	Magnetic nanoparticles	*Candida rugosa* lipase	Durability	[50]
Covalent bond	Epoxy resin ECR8285	SMG1-F278N lipase	Substrate selectivity	[13]
Covalent bond	Magnetic Cellulose Nanocrystals	*Pseudomonas cepacia* lipase	Substrate selectivity	[51]
Covalent bond	Agarose-based carriers/ cross-linked aggregates	*Candida rugosa* lipase	Substrate selectivity	[116]
Covalent bond	Chitosan beads	Lipase	Substrate selectivity	[117]
Covalent bond	Amino C2 acrylate	Arylmalonate decarboxylase	Substrate selectivity	[52]
Covalent bond	Calcium alginate beads	*Aspergillus aculeatus* polygalacturonase	Thermostability	[118]
Covalent bond	Glutaraldehyde CLEA	*Trametes versicolor* & *Fomes fomentarius* laccases	Thermostability and durability	[56]
Covalent bond	Glutaraldehyde CLEA	*Escherichia coli* lysine decarboxylase	Thermostability	[119]

Table 1. *Cont.*

Method	Support Material	Enzyme	Improved Enzyme Properties	Ref
IL based	poly(VEImBr)	CalB	Activity	[90]
IL based	Magnetic poly(ionic liquid) support	Cellulase	Activity and stability	[93]
IL based	Periodic mesoporous organosilica	α-Amylase	Thermostability	[94]
IL based	[Bmim][PF6]	*Candida antarctica* lipase B	Stability and reusability	[91]
SCF based	Supercritical fluids CO_2	Multienzyme	Productivity	[101]
SCF based	surface-modified mesoporous activated carbon	Horseradish peroxidase (HRP)	Thermostability and durability	[102]
SCF based	Chitosan–glyoxyl–EDA–glu	*Candida antarctica* lipase B	Stability	[103]
SCF based	Supercritical CO_2	*Candida antarctica* lipase B	Productivity	[100]
MOFs	PCN-333(Al)	HRP & Cyt c & MP-11	Higher affinity to Substrate and stability	[62]
MOFs	Enzyme@SNF@ZIF-8	*Penicillin G* acylase & catalase	Thermal/storage stability and durability	[72]
MOFs	ZIF-8	Carbonic anhydrase	Productivity	[68]
MOFs	IRMOFs	*Candida antarctica* lipase B	Activity	[69]
MOFs	ZIF-8	Cyt c	Activity	[70]
MOFs	csq-net Zr-based	Lactate dehydrogenase	Activity	[108]

4. Conclusions

We have summarized recent immobilization techniques, including adsorption-, covalent bonding- and MOF-based immobilization. Adsorption techniques based on van der Waals interactions and covalent bonding methods through covalent conjugation, which further affect the structure and substrate binding properties, may result in improvements in substrate selectivity. Most immobilization techniques can enhance the mechanical properties of structures or provide additional protection, making enzymes stable under thermal, acidic or toxic conditions. Moreover, immobilization can reduce product inhibition, which will help further affect productivity. According to more recent studies, combining multiple immobilization techniques with a tailored strategy for enzymes has become more popular in industrial applications.

The unique structure of MOFs helps enhance enzyme activity, generate a better diffusion rate and provide various options for enzyme immobilization. MOFs improve the properties of enzymes and thus have become a prospective platform for enzyme immobilization.

With the development of nanotechnology, an increasing number of nanomaterials has great potential for enzyme immobilization because of their unique properties, including high biocompatibility, large surface areas and other characteristics. Enzymes immobilized onto designed carriers with suitable linking groups are well developed. In the future, we believe that enzyme immobilization will be focused on consideration of the enzyme structure, carrier properties, and industrial needs.

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Abbreviations

CLEA	cross-linking enzyme aggregate
CLEC	cross-linking enzyme crystal
SCFs	supercritical fluids
ILs	ionic liquids
FAME	fatty acid methyl ester
MOFs	metal-organic frameworks
PGA	penicillin G acylase
ZIF-8	zeolitic imidazolate framework-8
OPAA	organophosphorus acid anhydrolase
LDH	layered double hydroxides
FalDH	formaldehyde dehydrogenase
FDH	formate dehydrogenase
GDH	glutamate dehydrogenase

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