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Review Article

Parameters Affecting the Performance of Immobilized Enzyme

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Enzyme immobilization has been investigated to improve lipase properties over the past few decades. Different methods and various carriers have been employed to immobilize enzyme. However, the application of enzymatic technology in large scale is rarely seen during the industrial process. The main obstacles are a high cost of the immobilization and the poor performance of immobilized lipase. This review focuses on the current status of enzyme immobilization, which aims to summarize the latest research on the parameters affecting the performance of immobilized enzyme. Particularly, the effect of immobilization methods, immobilization carriers, and enzyme loading has been discussed.

1. Introduction

Enzyme, which is produced from active cells, is a highly efficient catalyst. Compared with chemical catalyst, it has many advantages such as a high specificity, a high catalytic efficiency, and an adjustable activity, which greatly promote enzyme to be used in pharmaceutical, chemical, and food industries [1–4]. However, its poor stability, reusability, and high cost of single use limit its use in industrial production. In recent years, enzyme immobilization technology provides an effective method to circumvent these issues, which not only improves lipase catalytic properties and operational stability but also facilitates enzymes multiple reuse, separation, and continuously automatic operation in industrial production [5–7].

So far, various carriers and methodologies have been used for enzyme immobilization in order to improve the properties of free enzyme [8–11]. In fact, the performance of immobilized lipase relies on several important factors including immobilization methods, immobilization carrier materials, enzyme pretreatment before immobilization, and enzyme loading on the carriers. For instance, Knezevic et al. [12] discovered that coupling lipase via its carbohydrate moiety previously modified by periodate oxidation method had a high activity retention. Vaidya et al. [6] demonstrated that the porous AGE-EGDM polymer particles proved to be a better carrier for immobilization of *Candida rugosa* lipase than the porous GMA-EGDM copolymers due to the former having a larger specific surface area. Lee et al. [13] found that pretreatment of lipase with soybean oil before immobilization could prevent lipase activity loss when it was immobilized covalently on silica gel. Besides, our previous results suggested that the activity recovery and immobilized ratio had been influenced by the carrier surface properties [14]. Thus, exploiting novel immobilization methods and carrier materials have an important significance on enzyme immobilization. This paper provides a review of several important factors on affecting enzyme immobilization, including immobilization methods, immobilization carrier materials, and immobilization enzyme loading.

2. Effect of Immobilization Methods

Many methods have been established in order to achieve immobilized enzyme, and each has its advantages and defects. The methods used to date include physical adsorption, entrapment, covalent binding, and the immobilization via a spacer arm.

2.1. Enzyme Immobilization via Physical Adsorption. Adsorption immobilization is a method which is used to immobilize enzyme by the attachment of enzyme on carrier surface via

weak forces, such as van der Walls force, electrostatic force, hydrophobic interaction, and hydrogen bond [15]. Not surprisingly, the specific surface area of carriers is an important factor to influence the adsorption amount of enzyme. Recently, a new synthesized cyclodextrin-based carbonate nanosponge (CD-NS-1:4) was used for adsorption of *Pseudomonas fluorescens* lipase [16]. The results showed that the immobilized lipase presented high stabilization and good activity, even after two months of incubation at 18°C. Following the same method, *Candida antarctica* lipase B was adsorbed on macroporous resin NKA-9 (a polar macroporous resin produced by Chemical Plant of Nankai University, Tianjin, China) in organic medium and the immobilization conditions were optimized [17]. Consequently, the activity recovery achieved 83% with the mass ratio of lipase to support of 1 : 80 and immobilization time of two hours at 30°C. Compared with the free lipase, the immobilized lipase showed enhanced pH value and thermal stability. Also, Ponvel et al. [18] used magnetite particles modified with alkyl benzenesulfonate as carriers to immobilize *Porcine pancreas* lipase via physisorption. The immobilized lipase showed enhanced specific activity (8.7 U/mg) and durability in the reuse after recovery by magnetic separations. In addition, several other publications also reported that lipase immobilization via adsorption showed higher activity than free lipase [19, 20].

Physical adsorption immobilization is one of the simplest methods and can be conducted under mild conditions. This method does not result in large loss of enzyme activity. Despite many merits of adsorption, it also presents some drawbacks. For instance, the immobilized enzyme prepared by adsorption has poor operation stability; the amount of adsorbed enzyme is more susceptible to the immobilization parameters such as temperature, ionic strength, and pH; and enzyme can be stripped off easily from the carrier because of the weak forces between them.

2.2. Enzyme Immobilization via Encapsulation. For this immobilization method, the enzyme is entrapped in the internal structure of polymer material. In the study by Yang et al. [21], lipase from *Arthrobacter sp.* was immobilized by encapsulation in hydrophobic sol-gel materials. Under the optimum conditions, the total activity of the prepared enzyme achieved 13.6-fold of the free form. Moreover, the encapsulated lipase exhibited higher thermal stability and operational stability than the free lipase. Compared with covalently immobilized lipase, a higher percent activity yield of the encapsulated lipase (65 U/g) was obtained when *Candida rugosa* lipase was encapsulated within a chemically inert sol-gel support [22]. In addition, the encapsulated lipases had higher catalytic conversion in the hydrolysis of *p*-nitrophenyl palmitate and higher enantioselectivity in the enantioselective hydrolysis of racemic naproxen methyl ester than that of immobilized lipase prepared by covalent binding [22], which is probably because that this encapsulation immobilization preserves the mobility of the enzyme and allows to increase its activity and enantioselectivity.

2.3. Enzyme Immobilization via Covalent Binding. Covalent binding immobilization is a method which is used to immobilize enzyme by binding the nonessential pendant group of enzyme to the functional group of carrier via chemical bonds. It must be pointed out that the immobilization reaction to form the chemical bond should be carried out under mild conditions because the vigorous reaction conditions can destroy enzyme active conformation. Recently, functionalized Fe_3O_4 nanoparticles modified with carboxymethylated chitosan were developed and used as carrier for the covalent conjugation of papain [23]. Compared with the free papain, the magnetic immobilized papain exhibited good superparamagnetism, enhanced enzyme activity, better tolerance to the variations of medium pH and temperature, and improved storage stability as well as good reusability. More recently, Bai et al. synthesized a series of mesoporous and hydrophilic bead carriers containing epoxy groups and used them to immobilize glucoamylase (Glu) by forming covalent bond between epoxy groups and enzymes [24]. The activity recovery reached 86% and the immobilized Glus exhibited excellent stability and reusability than that of the free ones. In many cases, however, some of the carriers do not have functional group or the immobilization conditions are harsh if they are coupled directly. Therefore, enzyme cannot be coupled directly to the carriers. In order to reduce the loss of enzyme activity, the researchers thus often activate the carriers using some functional reagents before immobilization, which can make the enzyme immobilization conditions mild. For instance, using carbodiimide as coupling agent to activate the hydroxyl groups of chitosan, Chiou and Wu had successfully immobilized *Candida rugosa* lipase on chitosan [25]. Their results showed that the operational stability and storage stability of the immobilized lipase were enhanced greatly than that of free lipase. In addition, carbonyldiimidazole was also often used to activate the hydroxyl, carboxyl, and amino group in order to immobilize enzyme under mild condition [26, 27].

Generally, enzyme immobilization by covalent binding method can combine enzyme with carrier firmly and avoid the shedding and leakage of enzyme. However, the defect of this method is that it often causes the low activity recovery, which is resulted from the destruction of enzyme active conformation during immobilization reaction, the multipoint attachment to the supports, steric hindrance of enzyme, or the strong strength of the covalent binding.

2.4. Enzyme Immobilization via a Spacer Arm. Immobilization of enzyme on carriers via a spacer arm seems to be a good way to avoid the steric hindrance and to increase enzyme activity. This type of immobilization forms a spacer arm between enzyme and carriers by means of a bifunctional reagent such as glutaraldehyde and isocyanate. With the introduction of a flexible spacer arm onto the supports, the enzyme can be allowed to stretch flexibly and catch the substrate more easily. Isgrove et al. [28] reported that β-glucosidase and trypsin immobilized on carriers via polyethyleneimine who act as spacer arm exhibited an enhanced activity compared with the corresponding carriers without spacer arm. Recently, Bayramoğlu et al. studied the effect of coupling methods on the immobilized lipase [29]. They found that

Candida rugosa lipase immobilized onto poly(GMA-HEMA-EGDMA) microspheres (these microspheres were prepared via suspension polymerisation by using glycidyl methacrylate (GMA), 2-hydroxyethyl methacrylate (HEMA), and ethylene glycol dimethacrylate (EGDMA) as monomers) using 1,6-diaminohexane as spacer arm showed a higher loading capacity and apparent activity compared with the lipase directly immobilized via the epoxy groups. They thought that the increase in loading capacities and lipase activity was due to the spacer-arm effect. Kim et al. also investigated the effect of three different immobilization methods on lipase immobilized on functionalized silica nanoparticles [30]. They found that high loading capacity and high activity were gained when lipase was immobilized on ethylene diamine-activated silica nanoparticles via glutaraldehyde (GA) or 1,4-phenylene diisothiocyanate (NCS) as a coupling agent. More recently, Hu et al. employed magnetic Fe_3O_4 nanoparticles modified with 3-aminopropyltriethoxysilane as carriers to immobilize lipase from *S. marcescens ECU1010* (*SmL*) with glutaraldehyde as the coupling agent [31]. The immobilized lipase showed a higher binding efficiency and activity recovery than that of lipase adsorbed directly onto the supports. As well as, the similar results have been reported through introduction of aminopropyl spacer arm as *Bacillus licheniformis* L-arabinose isomerase (BLAI) was immobilized [32].

Many studies indicated that the spacer arm between enzyme and carriers could remove the enzyme away from the surface of carriers and prevent undesirable side attachment between enzyme molecules and support. This immobilization method favors the activity retention of immobilized enzyme and improves the performance of immobilized enzyme.

3. Effect of Immobilization Carriers

Another factor contributing a lot to the immobilized lipase is the immobilization carrier materials. Carrier material should be readily available, nontoxic, and should offer a good biological compatibility for enzyme [33]. As a part of the immobilized enzyme, the structure and property of the carrier have important impacts on the enzymatic properties. Several natural polymer materials and inorganic particles were commonly used as supporting materials [34–36]. Besides, many researchers had grown great concern in using various synthesized polymer materials as carriers for their good mechanical and easily adjustable properties [37–39]. Particularly, inorganic-organic composite materials have attracted deep attention [40–42].

3.1. Natural Polymer Materials. When it comes to the natural polymer materials, much attention has been paid to cellulose, chitin, chitosan, starch, and other natural polymer materials owning to their wide range of sources, easy modification, nontoxic, and pollution-free, with a variety of functional groups and good biocompatible properties. According to the previous study [43], *Candida rugosa* lipase which was immobilized on activated chitin by covalent attachment showed a higher thermal stability than that of the free lipase. Moreover, the immobilized lipase proved to be effective and reusable for synthesis of butyl esters. Another data reported by Hung et al. showed that immobilized lipase exhibited broader pH tolerance, higher heat stability, and still retained 74% residual activity after ten hydrolysis cycles when it was immobilized on chitosan [44]. In addition, Chiou and Wu carried out immobilization of *Candida rugosa* lipase on chitosan supports containing hydroxyl groups [45]. The resulting immobilized lipase enhanced the stability of lipase and exhibited excellent operational stability. And the prepared chitosan beads proved to be a good carrier for lipase immobilization.

3.2. Synthetic Polymer Materials. Synthetic polymer materials are prepared by chemical polymerization using various monomers. As a kind of important carrier, synthetic polymer materials exhibit the advantages of good mechanical rigidity, high specific surface area, easy to change their surface characteristics, and their potential for bringing specific functional group according to actual needs. Hence, they have been widely investigated and used for enzyme immobilization. Carriers which have large surface area always do a great help to obtain good immobilization efficiency. For instance, the macroporous polyacrylamide (PAM) microspheres which had a large surface area were synthesized by Lei and Jiang via inverse suspension polymerization and pectinase was covalently immobilized onto them [46]. The results showed that up to 296 mg of enzyme was immobilized per gram of the supports. Moreover, the immobilized pectinase displayed an improved thermal stability and storage stability over free lipase, and they also exhibited a better reusability than pectinase entrapped in alginate reported by Roy et al. [47]. That was possibly because PAM supports possessed many amino groups and they could provide a great number of available binding sites for immobilizing pectinase by covalent attachment. Li et al. selected polystyrene (PST) microspheres as carrier to immobilize lipase from *Burkholderia cepacia* and they researched the effect of the carriers' pore sizes on lipase immobilization [48]. They found that the specific activity of immobilized lipase had a close correlation with the pore size of the PST microspheres. The thermal and storage stabilities of immobilized lipase were enhanced with the increase in the support's pore sizes. It has also been reported that the property of the support was an important factor influencing the enzyme catalytic performance [49, 50]. In the study by Menaa et al., high loading capacity of protein could be obtained when carrier surface was regulated with hydrophobic groups [51]. To investigate the effect of carrier surface properties on lipase immobilization, Zhang et al. prepared a series of poly(vinyl acetate-acrylamide) microspheres with different hydrophobic/hydrophilic surface characteristics to immobilize *Candida rugosa* lipase [52]. Their results showed that hydrophobicity/hydrophilicity of the microspheres could influence not only the immobilized ratio but also the immobilized lipase activity. This was also pointed out in several other previous reports [53, 54]. Another publication reported that *Candida species 99–125* lipase was immobilized on macroporous polyglycidylmethacrylate beads via covalent binding [55]. It had been mentioned that crosslinker and porogen used in preparing the polymer beads could influence the porous structure of the polymer particles and thus affect enzyme immobilization. So the authors optimized their using amount

in the preparation of poly-GMA. The prepared immobilized lipase under the optimum lipase concentration showed a good pH stability, thermal stability, and reusability. In short, synthetic polymer materials can be obtained in various forms. Moreover, certain desirable functional groups can be introduced by polymerization using different monomers or modification by different groups.

3.3. Inorganic Magnetic Particles. Many magnetic particles have been used to immobilize enzyme with good results because they can provide some advantages such as small particle size, good superparamagnetism, and large specific surface area. Thus, great attention has also been aroused using inorganic magnetic particles to immobilize enzyme. Among many types of inorganic magnetic particles, nanoiron oxide has gained much attention for its efficient carrier properties and easy recovery with the aid of a magnet. It had been mentioned that functionalized γ-Fe_2O_3 magnetic nanoparticles were used as carrier to immobilize *Candida rugosa* lipase via chemical bond [56]. The prepared immobilized lipase showed long-term stability, which proved the significant advantage of Fe_2O_3 nanoparticles as enzyme immobilization carriers. Recently, lipase from *Serratia marcescens* has successfully been immobilized onto the amino-functionalized magnetic nanoparticles [31]. The results showed that the immobilized protein load could reach as high as 35.2 mg protein g^{-1} support and the activity recovery was up to 62.0%. Moreover, the immobilized lipase demonstrated a high enantioselectivity toward (+)-MPGM and it also displayed the improved thermal stability as compared to the free lipase. More recently, it has been reported that thioredoxin (Trx1) immobilized on iron oxide superparamagnetic nanoparticles with an additional silica shell coating before being treated with 3-aminopropyltriethoxysilane (APTS) via EDC (1-ethyl-3-{3-dimethylaminopropyl}carbodiimide) method showed good activity recovery and recyclability [57]. Compared with Trx1 immobilized on iron oxide superparamagnetic nanoparticles single coated with APTS(Trx1/APTES-MagNps), the doubly coated system exhibited more stability and could be recycled many times. The authors explained this to that an inhibitory site had been created after the first catalytic assay of the Trx1/APTES-MagNps.

3.4. Magnetic Polymer Microspheres. Magnetic polymer microspheres have been widely investigated not only because they possess favorable superparamagnetic properties facilitating its recovery from the reaction mixture, but also because they have good biocompatibility and various surface functional groups which are suitable to couple enzymes. In recent years, great efforts have been put into the studies of using magnetic polymer microspheres for enzyme immobilization. Liu et al. used methacrylate and crosslinker divinylbenzene to synthesize poly(methacrylate-divinylbenzene) magnetic microsphere in the presence of magnetic fluid via modified suspension polymerization [40]. The resulting magnetic microspheres exhibited superparamagnetism and they were used as supports to immobilize *Candida cylindracea* lipase. The results indicated that the immobilized lipase held high enzyme loading (34.0 mg g^{-1} support), high activity recovery (72.4%), and good stability during the repeated use. Moreover, the immobilized lipase showed better heat resistance and was more stable than the free one. In another work, epoxy-functionalized magnetic chitosan beads were prepared in the presence of epichlorohydrin via phase-inversion technique and they were used as carriers to immobilize *Trametes versicolor* laccase by covalent immobilization [41]. The measurement indicated that the magnetic chitosan beads had a large pore volume and a porous surface structure, which would favor high immobilization capacity for the enzyme. Furthermore, the experiments revealed that the thermal stability and storage stability of laccase were enhanced when enzyme was immobilized on the magnetic chitosan beads. Besides, immobilization of lipase from *Porcine pancreas* on magnetic Fe_3O_4-chitosan microspheres was also successfully achieved [42]. That study implied that microspheres synthesized with and without magnetic field (MF) had different morphology and surface area. The experiments illustrated that lipase immobilized on microspheres prepared under MF showed higher activity and better operational and storage stability than those immobilized on microspheres prepared without MF. In short, magnetic polymer material is a kind of excellent support for enzyme immobilization, and it will be widely used for its good biocompatibility and magnetic feature which enables it to achieve a rapidly easy separation from the reaction medium in a magnetic field.

4. Effect of Enzyme Loading

The excessive enzyme loading always causes protein-protein interaction and inhibits the flexible stretching of enzyme conformation, which will result in the steric hindrance and thus the inactivation of an enzyme. That is, the enzyme molecule may be difficult to modulate its most suitable conformation for catching the substrate molecules and releasing product molecules under molecular crowding condition. Recently, several authors have investigated the effect of enzyme loading on the immobilization [46, 52, 58, 59]. For instance, in the study of pectinase immobilization on macroporous polyacrylamide microspheres by Lei and Jiang [46], they found that the activity of the immobilized pectinase decreased instead when the enzyme amount increased from 10 to 12 units/mL. A similar result was also obtained by other research groups [58]. Hu et al. also discovered that an over-high amount of lipase adsorbed onto the support would make the lipase form an intermolecular steric hindrance and thus influence the performance of lipase [31]. Zhang et al. investigated the effect of steric hindrance on the immobilized lipase [52]. The experiments further demonstrated that an excessive lipase loading had resulted in an intermolecular steric hindrance and greatly affected the lipase activity. In addition, it was also shown by Xie and Ma [59] that the activity recovery of the immobilized lipase increased at the initial stage with the increase in enzyme amount from 1 to 5 mg per 200 mg carriers and then remained nearly constant with further increase in lipase amount to 9 mg. In general, the amount of enzyme immobilized on carriers has obvious influence on the performance of the immobilized enzyme. Therefore, we should pay enough attention to it as preparing the immobilized enzyme.

5. Conclusions

In all, many parameters will have influence on the properties of enzyme during enzyme immobilization. Particularly, immobilization methods, carrier materials, and enzyme loading amount have proven to be important for enzyme immobilization. Therefore, we should try to select a suitable carrier as well as an appropriate method for immobilization. Moreover, the immobilized enzyme amount could not be blindly raised in immobilization with the purpose of enhancing enzyme activity.

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