Recent progress in the development of immobilized penicillin G acylase for chemical and industrial applications: A mini-review

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ABSTRACT

Immobilized penicillin G acylase (PGA) as an important industrial catalyst can catalyze penicillin G potassium (PG) to 6-aminopenicillanic acid (6-APA). 6-APA is an important intermediate for semi-synthetic penicillin drugs, which occupies a huge market space in the anti-inflammatory field, as a result,

immobilized PGA occupies a huge market space in the pharmaceutical field. However, at present, there are different degrees of defects in the preparation and production process of immobilized PGAs on the market, because of the huge demand, therefore, the performance of immobilized PGA and its productivity will bring huge economic benefits to enterprises. Therefore, research on immobilized PGA has always been a focus. This review first introduces the source, classification, structure and catalytic mechanism of PGA, and then studies the development of immobilization methods, immobilized carriers, reaction media, enzyme activity regeneration and reactors of immobilized PGA in recent years.

1. INTRODUCTION

Since Fleming discovered penicillin in 1932, more and more penicillin drugs have been widely used in the medical field[1-3]. Penicillins are β-lactam antibiotics, because of their strong bactericidal activity, wide indications, low toxicity and good clinical efficacy, penicillins have become the most widely used class of antimicrobial chemotherapy drugs in clinic[4-6]. However, due to the large amount of wastewater discharged from the production of these drugs and the abuse of drugs, bacteria have developed serious resistance to them, which greatly weakens the efficacy of the original drug, resulting in the withdrawal of some β-lactam antibiotics from clinical practice[7, 8]. In order to slow down the emergence of drug-resistant bacteria and ensure the efficacy of drugs, 6-APA is now used as an intermediate to cope with semi-synthetic penicillin produced by derivatization of 6-APA[9-11]. However, 6-APA is mainly prepared from natural benzyl penicillin by chemical pyrolysis or enzymatic deacylation. The production of 6-APA by chemical method needs high toxic chemical reagents such as pyridine, PCl_5 and NOCl et al, which is easy to cause environmental pollution[12-15]. Compared with the chemical method, enzymatic method has many advantages, such as mild reaction conditions, high catalytic efficiency and avoided the use of toxic substances and so on[16-19]. The high efficiency, safety and low cost of enzymatic method provide a large amount of 6-APA for the synthesis of neo-penicillin, which

makes a breakthrough in the large-scale production of a series of neo-penicillins. As early as the 1950s, it was reported that PG was hydrolyzed by microorganisms to produce 6-APA and phenyl acetic acid[20-22]. Penicillin G acylase (PGA, E.C.3.5.1.11) as a kind of microorganisms was used to produce 6-APA because of its wide source, non-specific enzyme, less strict requirements on substrates and preferential hydrolysis of PG[23-25]. However, the enzymatic hydrolysis of PGA has its own shortcomings, which are mainly manifested in the instability of free PGA to heat, organic solvents and acid/base, which seriously affect the application of free PGA[26, 27]. In addition, free PGA is difficult to separate from the product, which not only reduces its reusability, but also increases the difficulty of separation and purification of 6-APA[28]. These problems have become a major bottleneck in the industrialization of PGA in the production of β-lactam antibiotics. To solve these problems, researchers used the method of immobilizing the PGA on the carrier to improve the environmental tolerance and the recovery performance by utilizing the properties of the carrier[29-31]. However, due to the limitation of immobilized technology, it was not until the 1970s that immobilized PGA was successfully used to hydrolyze PG to produce 6-APA[32], which replaced the traditional chemical pyrolysis method in industrial production.

In the process of catalyzing the production of 6-APA from PG by immobilized PGA, the recovery of enzyme activity, mass transfer rate, recovery and reusability of immobilized PGA are important indexes for evaluating the performance of immobilized PGA. The above performance is mainly affected by the following conditions, such as immobilization methods[33-35], immobilization carriers[28, 36-40] and immobilization process conditions[40-48] (such as reaction medium, carrier type, etc.). Although there are many reports on immobilized PGA, there are few reviews on immobilized PGA. So far, only a small amount of literature has reported on immobilized PGA, such as, Xue Ping et al. [49] summarized the immobilization of PGA by polymer carrier materials in 2005. Gong Junbo et al. [50] reported on the research progress of immobilized PGA reactors in 2005. Zhang Yewang et al. [51] reported on the

research progress of PGA immobilization method, reactor and reaction medium in 2008. Xu Li et al. [52] summarized the research progress on the methods of immobilization of PGA in 2010. However, above reports only give a narrow overview of immobilized PGA and are relatively old. With the rapid development of immobilized PGA methods and carriers with the improvement of PGA immobilization technology in recent years, it is necessary to give a comprehensive overview of immobilized PGA. In order to better understand the immobilization method, carrier, reaction medium and reactor design, the review first introduces the properties of PGA (such as source, classification, structure and catalytic mechanism), and then the development status of immobilization method and immobilized carrier, as well as the advantages and disadvantages of the immobilized method, carrier, reaction medium and reactor design are introduced, respectively. In addition, the challenges of immobilized PGA were analyzed and summarized, most importantly, the author provides some solutions, which may give peers some new ideas.

2. PENICILLIN G ACYLASE

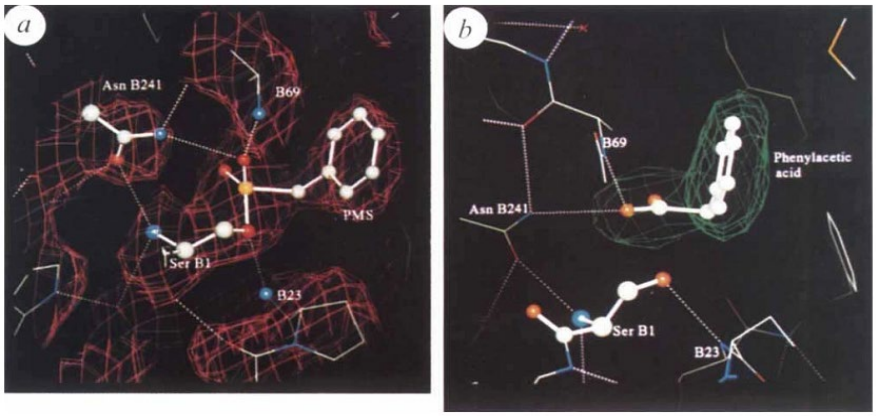
2.1. Source and Classification

Penicillin acylase is widely distributed in natural fungi, yeasts, actinomycetes, bacteria and so on[53]. Although the penicillin acylases produced by these producing bacteria can catalyze the same reaction, the substrate specificity and active centers of the above penicillin acylases from different sources are obviously different[54]. According to the priority of substrates in catalytic hydrolysis reaction, there are three main types: penicillin G acylase, penicillin V acylase and amino penicillin acylase. Among the penicillin G acylase, E.C.3.5.1.11 is the most widely produced and applied variety, and its structure and function are also the most in-depth study. The results showed that E.C.3.5.1.11 was mainly produced by bacteria (Escherichia coli)[54]. The molecular weight of E.C.3.5.1.11

(Hereinafter referred to as PGA) was 67000 ±1000, the specificity of the substrate was not strictly required and they belonged to non-specific acylase, but it can preferentially hydrolyze PG[55].

2.2. Structure

PGA is composed of two subunits, which is α and β[56, 57]. The two subunits are closely intertwined by hydrogen bonding and assembled into a kidney shape with a groove in the middle. The size of the heterodimer is 7nm×5nm×5.5nm[56]. There is no distinct separation between them, which contains 20% hydrophobic amino acids. The α and β subunit alone do not have enzymatic activity and are only active when they are combined in an appropriate form. The α subunit with a molecular weight of 20-23 kDa contains a side-binding site, which determines the specificity of the PGA to the substrate. The N-terminal serine of the β-subunit of 65-90 kDa is the only active center for its catalytic conversion, each functional amino acid residue in the vicinity of β-subunit contains various functional groups related to catalysis. The interlaced grooves of α and β subunit have a hydrophobic bag, **Figure.1** shows the hydrophobic bag structure of PGA, Metαl42, PheA146, PheB57, TrpB154, IIeB177 are located on both sides of the hydrophobic bag, SerB67 seals the bottom pocket of the hydrophobic bag, and the inlet is composed of side chains of SerB1 and AsnB241 and main chain nitrogen atoms of B23 and B69[56]. The structure of the hydrophobic bag plays an important role in maintaining enzyme activity.



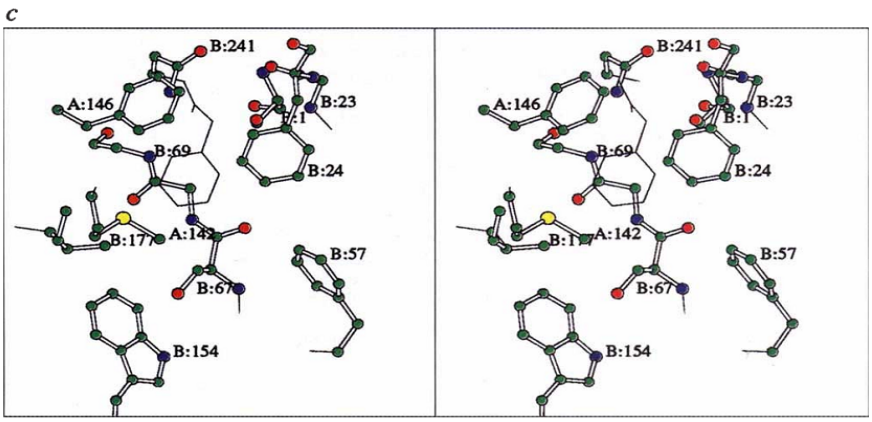


Figure.1 The active site structure of complexes of PGA. a. The product of the reaction of phenylmethylsulphonyl fluoride(PMSF) with PGA. It shows the covalent modification of the side chain of serine B1 to form phenylmethane sulphonyl O-serine(labeled PMS and Ser B1). The interactions of this analogue to one of the tetrahedral states in the enzymatic reaction with the main-chain nitrogens of B23 and B69 and the side chain of asparagine B241 are shown. Dotted lines represent possible hydrogen bonds. b. A similar view of difference density for the complex of PGA with phenylacetic acid showing Ser B1 poised for nucleophilic attack at the carbonyl carbon. c. Stereo view of phenylacetic acid in the binding pocket. View is approximately that from the righthand side of a and b. The phenylacetic acid molecule is shown as a line drawing and the nearby residues as 'ball-and-stick' representations. [56] (Duggleby et al. Copyright 1995, Springer Nature)

2.3. Catalytic Mechanism

In the hydrolysis process (the hydrolysis mechanism of PG by PGA is shown in **Figure.2**), B-1 serine residue in PGA molecular chain structure plays an important role in hydrolyzing amide bond. In the enzymatic hydrolysis of PG, the active center of PGA reacts with PG through nucleophilic substitution to form a tetrahedral intermediate, which can form and disintegrate reversibly. The protonation of the carboxyl group in the active center will facilitate the nucleophilic reaction of PG carbonyl carbon atoms. At the same time, the residue of B-1 serine is in the position, where it is easy to attack the carbonyl carbon atoms. However, there are no histamine and free carboxyl groups near the residue of B-1 serine.

To improve the nucleophilic ability of the carboxyl group, it is necessary to have an alkaline group in its adjacent position. As far as the active center of PGA is concerned, the only basic group of PGA is the A-amino group provided by serine. Although A-amino groups can interact directly with carboxyl groups on serine residues, it requires a water molecule to act as a bond bridge to regulate the alkalinity of A-amino nitrogen atoms. The isoelectric point of free amino groups in proteins is generally 6.8-7.9, so the charge less. A-amino group can directly accept a proton from the water molecule, so that the water molecule acts as a base and accepts the proton provided by the serine carboxyl group. More importantly, there is a hydrogen bond network structure on the active site of the PGA catalysis, which can fully support the role of water molecules as bond bridges. In this way, serine can form three hydrogen bonds related to tetrahedron, and the nitrogen atom of A-amino group can also form a hydrogen bond related to tetrahedron. These interactions can lead a pair of lone pair electrons on the non-charged amino nitrogen atom to the water as a bond bridge, which facilitates the transfer of protons from serine through the bond bridges formed by water molecules to the A-amino group.

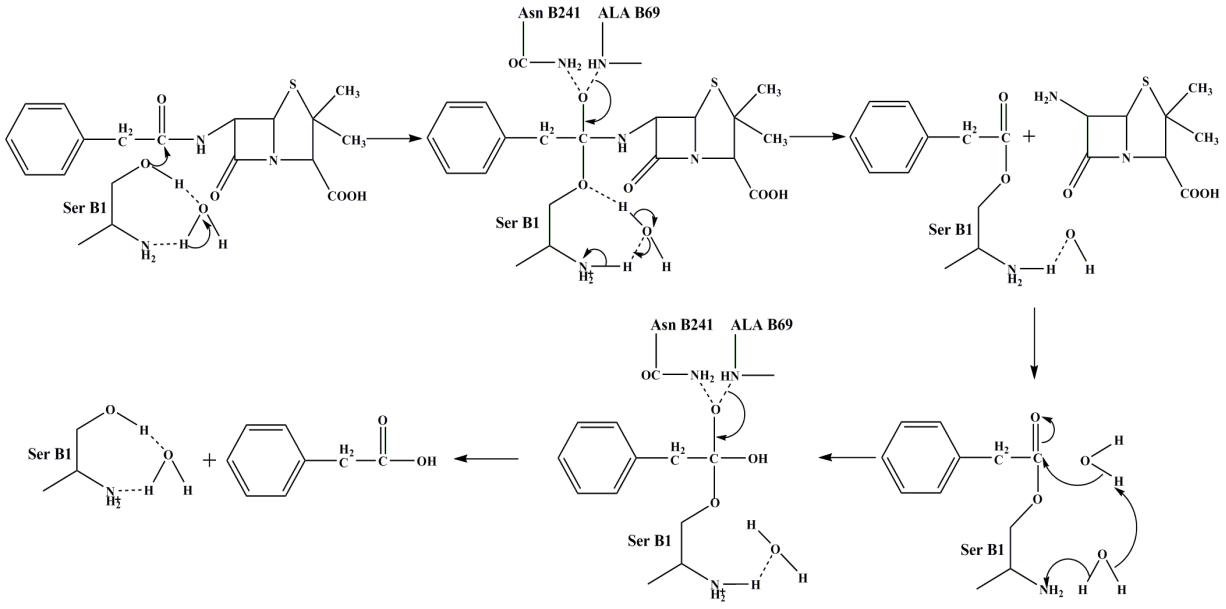


Figure.2 Hydrolysis mechanism of PG catalyzed by PGA. Steps in the proposed catalytic mechanism showing the roles of the nucleophilicity of serine B1 and showing the interactions that form the oxyanion by nucleophilic attack

by a water acting as a virtual base, or deacylation could be the result of direct nucleophilic attack by water interacting with the α-amino group. The interaction of the seryl 0γ and the main-chain nitrogen of B23 is not shown and is assumed to remain throughout the reaction[56] (Duggleby et al. Copyright 1995,Springer Nature)

3. IMMOBILIZATION PGA

3.1. Immobilization Method

The spatial configuration of PGA active center is the essential factor for its catalytic activity. Therefore, in the process of immobilization, reducing the conformational damage of PGA is important in maintaining the PGA activity. However, the selection of immobilization methods is often related to the structure of the carrier and the types of functional groups. Because of the different structure and types of functional groups of the carrier, the binding modes of the PGA to the carrier are also different, and the binding modes have a significant impact on the reusability and activity of the immobilized PGA. At present, the methods for immobilized PGA can be divided into the following four types: adsorption method, embedding method, cross-linking method and covalent binding method.

3.1.1. Adsorption Method

The adsorption method includes an ion adsorption method and a physical adsorption method, and the immobilization method generally used for PGA is a physical adsorption method, which is the immobilization process of PGA by using weak interaction between PGA and carrier molecules, such as hydrogen bond, electrostatic interaction, etc[58, 59]. This method is usually applied to immobilize PGA on porous or multi-layered materials with large specific surface. Because physical adsorption method is through weak interaction of non-chemical bonds to realize the immobilization of PGA, the effect on the conformation of the active center of PGA is relatively small, so the recovery rate of immobilized PGA activity is higher[60]. However, due to the weak binding force between immobilized PGA and carrier,

PGA not only can not improve its environmental tolerance with the aid of the carrier properties, but also can easily fall off in the operation process, and cannot be reused many times[61]. Fang Huan et al. [62] immobilized PGA on porous ceramic membranes by physical adsorption method showed that the activity of immobilized PGA was only about 50% when they were stored at the optimum catalytic temperature for 4 h. Xue Ping et al. [63]studied the operation stability of immobilized PGA adsorbed on MCM-41, it was found that the activity retention rate of immobilized PGA was only about 55% after six batches of operation.

3.1.2. Embedding Method

Embedding method is a common method in the early research of immobilized PGA. It embeds PGA in the carrier, and realizes the reuse performance of immobilized enzyme by improving diffusion resistance and extending the movement distance of PGA[64]. In principle, any material that does not

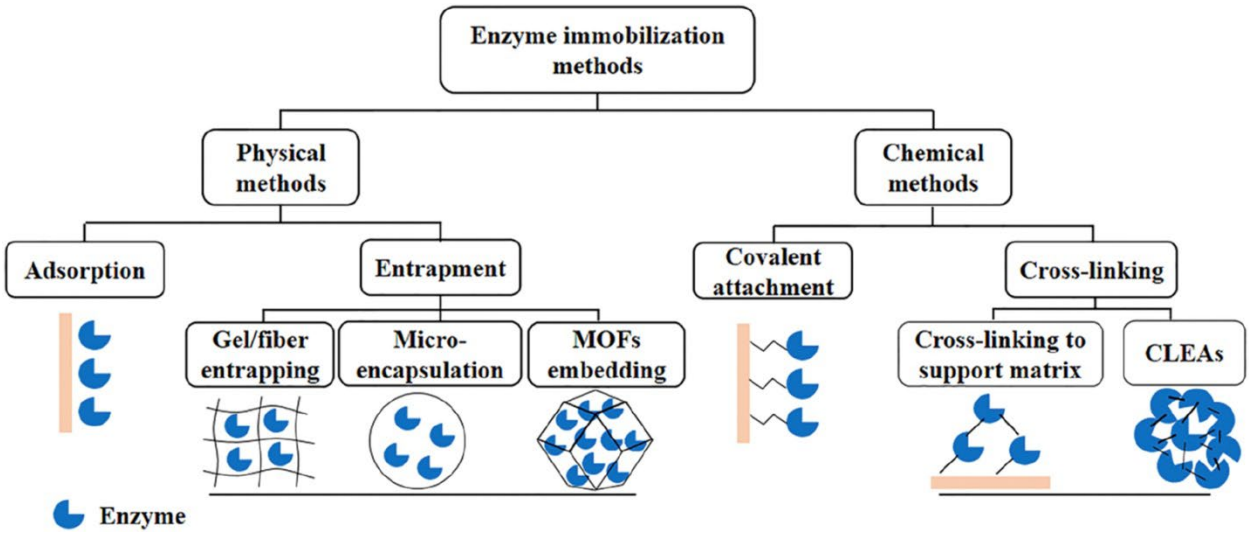


Figure.3 The methods for enzyme immobilization[65] (Dong-Mei Liu et al. Copyright 2018, Elsevier).

involve high temperature preparation and molding process can be used as an immobilization carrier in this method. Considering the fact that dense structure will make substrate diffusion difficult, the research on embedding method mainly focuses on amorphous macromolecule materials with loose

structure and good permeability[66]. Embedding methods include fiber embedding, gel embedding and microencapsulation. This method is simple in operation, mild in reaction conditions, and has little effect on the conformation of enzyme molecules, so the recovery rate of PGA activity is high. Most importantly, because PGA is embedded in the carrier, compared with the adsorption method, the immobilized PGA is not easy to fall off and lose in the process of operation, so the reusability is relatively good. However, it is also because PGA is embedded in the carrier, which leads to a slow diffusion rate and difficult separation of products and substrates, which reduces the catalytic efficiency of the immobilized PGA[67]. Norouzian et al. [68] filled the immobilized PGA into the packed column by embedding method to react in batches, results showed that the activity of PGA could only be maintained in 5 batches (9 h), which could be attributed to the PGA being easy to fall off during operation. Gist company[49] used gelatin and chitosan to encapsulate PGA-producing Escherichia coli, DSM antibiotic brand assemblase, for the enzymatic synthesis of β-lactam antibiotics, and used the active site titration method to measure the hydrolysis of penicillin, the result was that the rate of Kcat of immobilized PGA is $22S^{-1}$, which was much lower than that of free PGA ($66\ S^{-1}$). Unlike the PGA immobilized on the surface of silica gel, Basso et al. [49]embedded PGA in the silica aerogel and obtained the apparent activity recovery rate was only 10%, which could be attributed to the diffusion limitation inside the gel medium, while PGA itself was very sensitive to the diffusion limitation.

3.1.3. Cross-linking Method

Cross-linking method is a carrier-free immobilized enzyme method, which uses cross-linking agents to cross-link PGA. Covalent bonds were formed between PGAs and cross-linkers, and the structure of cross-linking framework was obtained[35, 69]. In addition to cross-linking between PGAs, there were some intramolecular cross-linking. This immobilized PGA has a high unit concentration of PGA, which can effectively avoid the problem of "dilution of enzyme activity" of immobilized PGA on the carrier, and

has a high catalytic efficiency[33, 70]. However, because cross-linking occurs between PGAs, which easily destroys the catalytic sites of PGA, leading to low activity recovery and poor filling ability of immobilized PGA. Therefore, the cross-linking method is often used as an auxiliary means of adsorption method or embedding method in the immobilization process, which is not used alone[71]. For example, by the adsorption method and the cross-linking method combined with the immobilized PGA, compared with the adsorption method, since the cross-linked PGA has a large volume and is not easily detached, the reusability of the immobilized PGA can be effectively improved. Compared with the cross-linking method, the density of the immobilized PGA is small, and the reaction area can be effectively increased. However, PGA immobilized by adsorption-cross-linking tends to still face the problem of a low mass transfer rate[72].

3.1.4. Covalent Binding Method

Covalent binding is one of the most commonly used methods in the study of PGA immobilization. It uses the covalent binding by the functional groups between PGA (In general, the functional groups available for attachment on the enzyme molecule are: α-amino or ε-amino; α-, β-, γ-hydroxy; thiol or carboxyl; imidazolyl; phenolic, etc.) and the carrier (-SH、-CN、-NH2、-CH2=CH2、-COOH、-CHO et al) to achieve the immobilization of PGA[73]. Because the covalent binding method immobilizes PGA through the reassembly of chemical bonds, the binding is firm and conducive to repeated recycling. However, due to the different types of functional groups binding to PGA[74], the active site conformation of immobilized PGA may be destroyed to varying degrees, which will lead to the decrease of PGA activity[75]. Therefore, how to further improve the activity of immobilized PGA by covalent binding method (i.e. on the premise of ensuring the strong binding of immobilized PGA) has been an important research direction of researchers. Owing to the reaction of epoxy group with amino group under mild conditions, researchers in the field of immobilization have paid much attention to it[76, 77].

It was found that when the epoxy target designed in the carrier was combined with the amino group in PGA, the PGA activity center conformation was less likely to be destroyed[28, 78]. Therefore, in recent years, epoxy groups have been mostly used as targets for immobilization of PGA. Mateo et al. [79] immobilized PGA on epoxy carrier by multi-point covalent binding method, it was found that covalent binding method could effectively stabilize the structure of the PGA, thereby improving the catalytic activity and activity stability of PGA. Covalent binding method has been industrialized as the most commonly used method for immobilized PGA.

3.2. Carrier type

The form of immobilized carrier for PGA can be divided into two types: carrier-free PGA and PGA with carriers. The choice of the immobilization carrier often needs to combine the performance of the PGA and the structure of the carrier.

3.2.1. Carrier-free PGA

Carrier-free PGA includes two types, cross-linked enzyme crystals (CLECs) and cross-linking enzyme aggregates (CLEAs), respectively.

CLECs were first proposed in 1964, but their use as biocatalysts was in the 1990s[80, 81]. The manufacturing process mainly comprises two steps: crystallization treatment of the concentrated enzyme solution, and then crosslinking treatment using a multifunctional reagent such as glutaraldehyde[81]. The cross-linked enzyme crystal has the advantage that the PGA concentration in the crystal lattice of the PGA, is close to the theoretical limit due to the cross-linking reaction using pure PGA. The forces that maintain the crystal structure of PGA are mainly hydrophobic and electrostatic. When PGA is converted from a solution environment to a crystalline environment, the electrostatic and hydrophobic effects of PGA is significantly increased. It maintains its stability against heat and other denaturing conditions by preventing its unfolding, aggregation and dissociation, allowing it to withstand

high temperatures, extreme pH, denaturation of organic solvents or other denaturing agents, and degradation of proteases[82-84]. The freeze-dried products only need to be stored at room temperature, and the mechanical strength is also significantly enhanced, with certain chemical and mechanical stability. Because there is no inert carrier in CLECs, the content and purity of PGA is high, which improves the reaction speed and specificity of enzymes. CLECs exist in solid form and are insoluble in water and organic solvents, therefore, they are easy to recycle. At the same time, they are easy to recover and regenerate. It's reported that the cross-linking enzyme crystal of PGA (T-CLECs) retains 70% of its initial activity after 1000 batches of reactions[85]. However, because the crystallization of concentrated PGA solution is difficult, it is the key for CLECs to be widely used, because the crystallization process usually takes a long time, and the workload is huge. Altus Biologics introduced commercial penicillin G acylase cross-linking enzyme crystals (CLEC-EC), which have been discontinued now[85].

Unlike the crystallization process of CLECs, CLEAs promote the polymerization of PGA by adding salts, organic solvents and high molecular polymers, and promote the deposition of PGA by using ammonium sulfate, polyethylene glycol, ethanol, and the like. The sedimentation is actually a protein purification process, and then a cross-linking agent such as glutaraldehyde is used to crosslink the crystallization PGA to obtain an insoluble and stable immobilized PGA[86, 87]. In extreme environments such as high temperature, strong acid and alkali, the stability of the enzyme in the organic solvent is greatly improved. CLEAs show good activity in organic media and have good application prospects in non-aqueous phase enzyme catalysis[88, 89]. Cao et al.[90] used ammonium sulfate, tert-butanol and PEG 8000 as precipitants respectively, and prepared PGA-CLEAs using glutaraldehyde as a crosslinking agent, and found cross-linking obtained using tert-butyl alcohol as a precipitant, PGA-CLEAs have the highest activity. However, although the CLEAs has the above advantages, it has the same problems of

high operation technology, high difficulty, long process time, and low effective utilization rate in the operation process as CLECs. Therefore, the application in the actual production process is limited.

As for the PGA with carriers, because the activity, recovery, response rate and reusability are important indicators for evaluating the performance of immobilized PGA, and they are closely related to the nature of PGA and the carrier. Therefore, the composition and structure of the immobilized carrier are the key factors affecting the performance of immobilized PGA[3, 38, 39]. As a result, how to design a carrier with suitable composition and structure has been the focus of the field. At present, according to the composition of the carrier, the immobilized carrier can be divided into four types: inorganic carrier, organic carrier, composite carrier and new carrier.

3.2.2. Inorganic carrier

Inorganic carriers have been widely used in the immobilization of PGA because of their high mechanical strength, strong corrosion resistance, large specific surface area, uniform pore size distribution, and low cost et al[39, 91]. Among them, mesoporous molecular sieves are a key point in the study of PGA immobilization carriers in inorganic materials[92]. Their specific surface area is about 1000 m^2/g, pore size is 2-50 nm and pore volume is about 1.0 cm^3/g[93]. As a carrier of PGA, they can not only effectively increase the load of immobilized PGA, but also effectively improve the response rate of immobilized PGA (also known as mass transfer rate)[94, 95]. However, although its surface is rich in hydroxyl groups, there are few functional group types, which limits the method of immobilization of PGA. Therefore, most researchers use physical adsorption to immobilize the enzyme. Although this method has the advantages of simple operation, low cost and high immobilization activity, the binding force between PGA and carrier is not firm and can easily fall off during operation, which seriously affects its reusability[95, 96]. To solve the above problems, researchers grafted functional groups on inorganic

materials by chemical modification, such as - SH, - CN, - NH_2, - CH_2 = CH_2, -COOH, - CHO, etc., to immobilize PGA by covalent binding with functional groups in PGA[97-101].

Xue Ping et al[102] studied the stability of MCM-41 immobilized PGA, it showed that after 6 batch operations, the viability retention rate was 55%. The immobilized PGA activity was higher, but the operation stability was poor. The reason is that a functional group such as -NH_2 on the surface of the PGA forms a weak hydrogen bond with -OH in carrier and PGA is liable to fall off. In addition, the by-product phenyl acetic acid formed after hydrolysis of PGA is a acid, which easily forms a locally highly acidic microenvironment in the pores of MCM-41, partially deactivating PGA. Later, they

Table 1 Immobilized carrier and immobilized method

Carrier Type	Carrier	Method	Ref.
Inorganic Carrier	MCM-41	Adsorption method	[103]
	TiO_2	Crosslinking Method	[104]
	SiO_2	Covalent Binding	[39]
	Magnetic $Ni_{0.5}Zn_{0.5}Fe_2O_4$ nanoparticles	Adsorption method	[105]
Organic Carrier	Chitosan–Resin (Ip-Csr)	Crosslinking Method	[106]
	Poly(vinyl acetate-co-diviyl benzene)	Crosslinking Method	[37]
	Highly Porous Cellulose-Based Polymeric Membrane	Crosslinking Method	[107]
	Poly(GMA-co-EGDMA)	Covalent Binding	[108]
	Agarose	Crosslinking Method	[109]

	Polyacrylonitrile fibres	Adsorption method	[110]
	Polyacrylic acid	Crosslinking Method	[111]
	PDEA-b-PHEMA-b-PGMA	Covalent Binding	[112]
	PDEA-b-PHEMA-b-P(MMA-co-GMA)	Covalent Binding	[113]
Magnetic Nano-Composite Carrier	Fe_3O_4/SiO_2/GAMM	Covalent Binding	[28]
	Magnetic Poly(HEMA-co-Diviyl Benzene)	Covalent Binding	[114]
	Magnetic Poly(GMA-co-MBAA)	Covalent Binding	[115]
	Fe_3O_4@Sio_2–NH_2/PAMAM	Covalent Binding	[116]
	Magnetic Poly(GMA-MBAA-NVP)	Covalent Binding	[117]
	Magnetic Poly(GMA-HEMA-MBAA)	Covalent Binding	[118]

continued to study the M41S series of mesoporous molecular sieve immobilized PGA [38-40], by introducing metal ions such as Al, Fe and Co on the surface of mesoporous molecular sieves, increasing the hydroxyl content of the carrier and PGA was immobilized by adsorption method. The results showed that the introduction of above metal ions can effectively increase the capacity loading, catalytic efficiency of PGA, but the operation stability is poor. In order to improve the binding force between PGA and carrier and prevent PGA from falling off, Xue Ping et al. [96, 119] further studied that after the metal-supported carrier silane was coupled, PGA was covalently cross-linked with glutaraldehyde, results showed that the operational stability of the immobilized PGA is improved, but because the pore size of the molecular sieve was significantly reduced, which reduced the capacity loading of PGA, and the activity of the immobilized PGA was also lowered accordingly. Besides, the mass transfer rate of the immobilized PGA is further reduced due to the modification of its surface and the reduction of its pore size, which has become a major bottleneck in the study of mesoporous molecular sieve immobilized PGA.

3.2.3. Organic Carrier

Compared with inorganic carriers, organic carriers have many kinds of functional groups, it is also easy to realize the design of carrier targets and micro-environments, which makes the immobilized PGA activity higher. Organic carriers include natural polymers and synthetic polymers. Natural macromolecule carriers mainly include cellulose[107], dextran[120-122], agarose[123], chitosan[124, 125] and so on. Because of their wide sources, pollution-free, renewable and other advantages, they have attracted the attention of researchers. However, with the deepening of research, it can be found that the following common defects of natural polymer materials hinder their application: firstly, when natural polymer is used as carrier, it needs to be pre-activated, which increases the difficulty of synthesis process; secondly, for different kinds of natural polymers, due to their different sources, they also have different properties. For example, cellulose, dextran and other materials after activated treatment often lead to the decline of the enzyme activity of immobilized PGA. Agarose has strong ability to bind enzyme, it can prepare immobilized PGA with high activity and stability, however, its mechanical properties are poor, which is not conducive to reuse of agarose[126, 127]. When chitosan is used as carrier, because of the limitation of its functional group, PGA is immobilized by adsorption method, which is easy to fall off, resulting in poor operational stability[60, 128]. In order to overcome the above shortcomings of natural macromolecule materials, researchers have developed synthetic macromolecule resin as the carrier of PGA. Compared with natural polymer carriers, synthetic polymer resin carrier has the advantages of strong mechanical properties, controllable functional groups and so on. These resins can be divided into polyacrylonitrile gel[129, 130], polyacrylamide gel[131-134], polyacrylic acid[111, 135] and polymer containing epoxy group[73, 76]. However, most of the synthesized macromolecule resins still have different defects as immobilized PGA carriers. For example, polyacrylonitrile is a very fine fiber, which is difficult to separate from the substrate and products, seriously affecting its reusability[136, 137]. Polyacrylic acid gel as immobilized PGA carrier was not conducive to the diffusion of substrates and

products, resulting in slow response rate[138]. However, after studying a large number of macromolecule resin carriers, researchers made an important discovery: macromolecule polymers containing epoxy groups can be opened around 25^{o}C and directly covalently bonded with amino groups of PGA[28, 139]. As the carrier does not require pre-activation, the operation procedure is simplified. Moreover, due to the mild reaction conditions, the immobilization process can reduce the loss of catalytic activity of PGA caused by harsh reaction conditions[28, 140].

Besides, in view of the above-mentioned mesoporous molecular sieves having a large pore size and a large specific surface area, researchers have prepared a mesoporous microsphere as a carrier for immobilized PGA[141], which mainly has cross-linkers composed of a cross-linking agent and functional monomers. Compared with mesoporous molecular sieves, it has many advantages such as many kinds of functional groups, controllable target number and so on[142, 143]. The cross-linked mesoporous polymer microspheres are widely used in immobilized PGA carriers, and are currently the most widely used in the market. Eupergit C is a granular cross-linked polymer with an average pore size of 23 nm, it is the most widely used immobilized PGA carrier in the market. However, it still exists some defects such as slow response rate, ineffective separation of products and substrates[73, 144-146].

According to the reports, Wang Weichen[147] first compared and studied the adsorption state of lipase in mesoporous microspheres, macroporous microspheres and planes, and found that macroporous microspheres have higher loading and mass transfer rate than the other two types of carriers. Then, they clarified the main reasons for higher activity of immobilized enzymes are lower pore curvature, larger pore space, multilayer state of adsorbed enzymes and better mass transfer effect by regulating the adsorption time and enzyme concentration. Kneževic´-Jugovic´ [108]et al. synthesized macroporous poly(glycidyl methacrylate-co-ethylene glycol dimethacrylate) [poly(GMA-co-EGDMA)] as carriers for immobilized PGA, its average pore diameter is about 151 nm, which is a kind of macroporous microsphere, almost 6 times as large as the Eupergit C pore diameter (**Figure.4** showed SEM micrograph

of poly(GMA-co-EGDMA) and Eupergit C). Besides, in combination with the immobilization method (as shown in **Figure. 5**), the reason for the better stability of poly(GMA-co-EGDMA) as PGA carrier than that of Eupergit C was analyzed, optimization of PGA immobilization on poly(GMA-co-EGDMA) was continued by investigation of the effects of different ionic strengths (0.5–1.5 mol/L) on the enzyme coupling yield and activity yield at the previously determined optimal pH. Immobilization of protein on epoxy carriers is usually conducted at high ionic strengths as it reduces adsorption based on electrostatic interactions and promotes enzyme adsorption via hydrophobic residues, which is followed by slow formation of covalent bond between carrier epoxy groups and enzyme by a ring-opening reaction. This synthetic copolymer could be a very promising alternative for the development of low-cost, easy-to-prepare, and stable biocatalyst compared to expensive commercially available epoxy carriers such as Eupergit C by optimizing the copolymerization process and the enzyme attachment procedure.

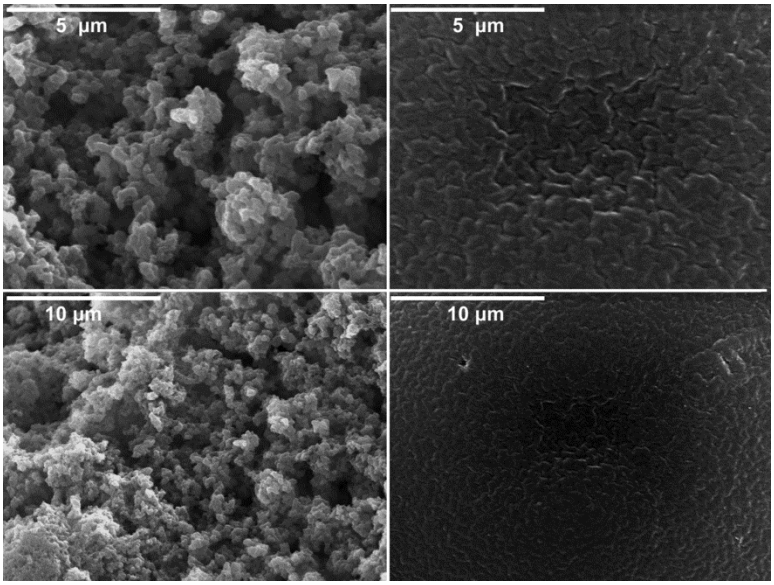


Figure.4 SEM micrograph of the cross-section of poly(GMA-co-EGDMA) at scale bar (a) 5 and (c) 10 µm and the cross-section of Eupergit C at scale bar (b) 5 and (d) 10 µm[108] (Kneževic´-Jugovic´ et al. Copyright 2015, John Wiley and Sons)

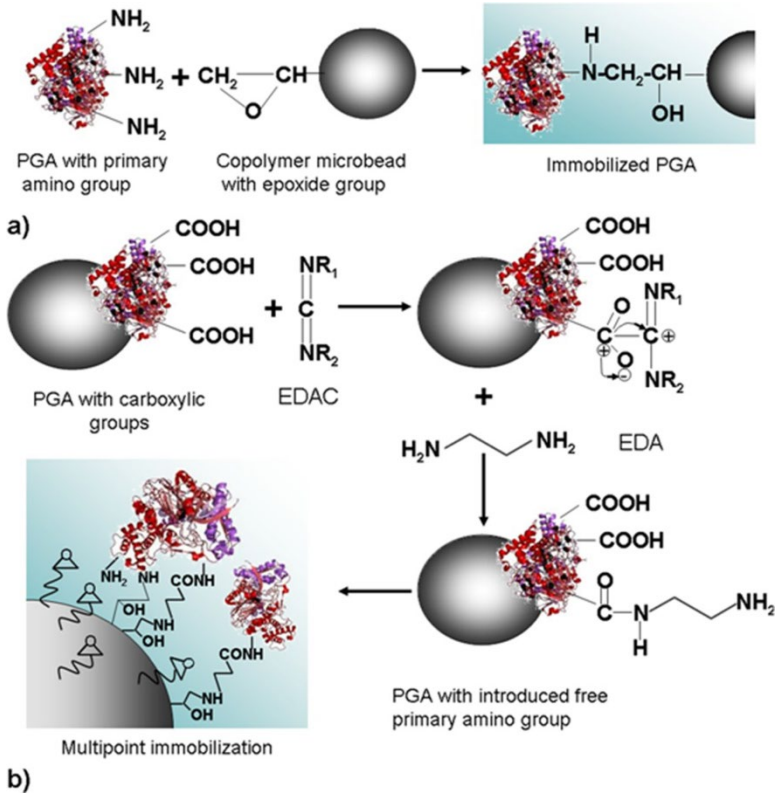


Figure.5 Schematic representation of (a) conventional method for PGA immobilization on epoxy carriers based on direct enzyme binding on the polymer via epoxide groups in one step and (b) modification of the procedure including post-treatment consisting of the chemical amination of PGA with ethylenediamine (after activation of the carboxylic function with carbodiimide)[108] (Kneževic´-Jugovic´ et al. Copyright 2015, John Wiley and Sons)

3.2.4. Composite Carrier

In recent years, researchers have developed a composite carrier[40, 106], which combines the advantages of organic and inorganic carriers, such as a large specific surface area, stable physicochemical properties (strong acid and alkali corrosion resistance), many types of organic carrier functional groups and so on. In composites, because the magnetic nanoparticle composite carriers have strong magnetic responsiveness, which can be easily separated from the reaction system, as a result, it has the advantages of easy recovery and good reusability compared with other carriers [40, 74, 148],

therefore, it is an important research object in the current PGA immobilization carrier. However, in the process of synthesizing magnetic nano-composite carriers, due to the easy aggregation of magnetic materials, in order to improve the dispersion of magnetic particles, the reverse phase suspension polymerization method is mostly used at present[148].

Xing Chen et al.[28] synthesized paramagnetic polymer microspheres by the inverse suspension polymerization method through polymerization of glycidyl methacrylate, ally glycidyl ether and methacrylamide on the surface of silica-coated Fe_3O_4 nanoparticles using *N*,*N*'-methylene-bis(acrylamide) as a cross-linking agent. PGA was covalently immobilized on the surface of the paramagnetic microspheres by reacting the amino groups of the PGA molecules with the epoxy groups of the paramagnetic polymer microspheres. Results showed that immobilized PGA on the paramagnetic polymer microspheres shows a high initial activity of 430 U/g (wet) and retains 99% of its initial activity after being recycled 10 times. Furthermore, immobilized PGA exhibits high thermal stability, pH stability and excellent reusability, which can be rapidly recycled by the aid of magnet(**Figure.6**).

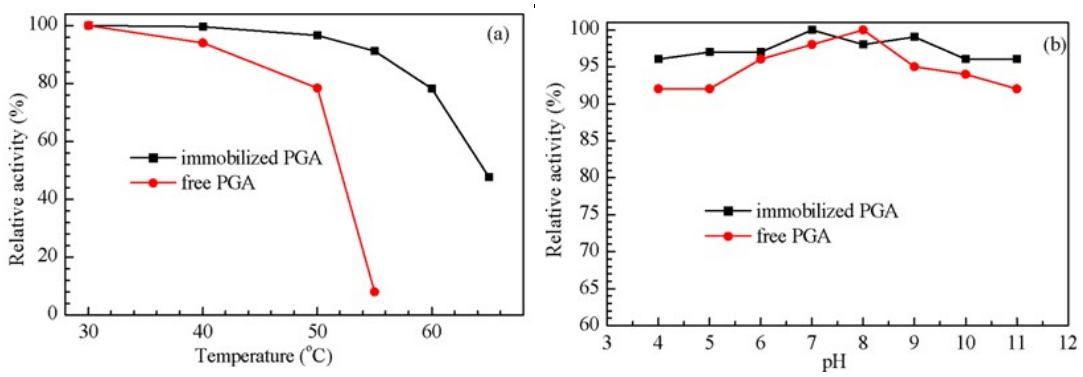


Figure.6 The thermal stability (a) and pH stability (b) of free and immobilized PGA on the paramagnetic polymer microspheres[28] (Xing Chen et al. Copyright 2018, Elsevier)

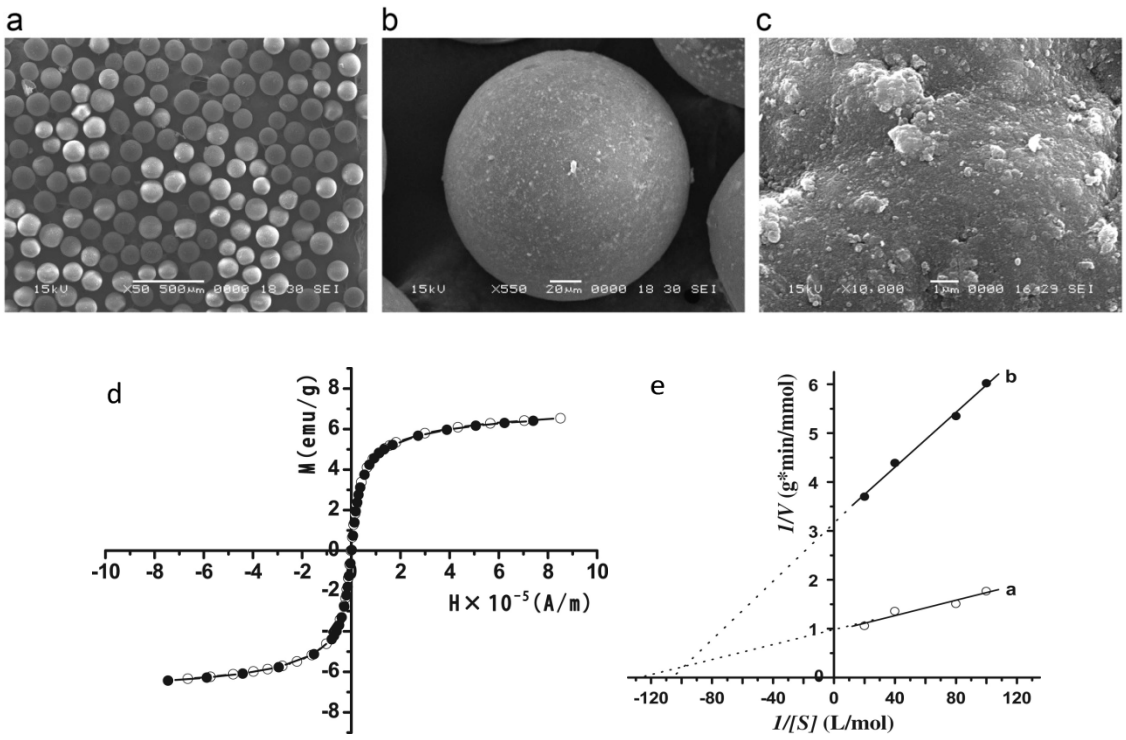


Figure.7 SEM images of the magnetic poly(GMA-MBAA-NVP) microspheres (a)×50, (b)×550, and (c) ×10,000. (e): Magnetization curves of magnetic poly(GMA-MBAA-NVP) microspheres; (d): Lineweaver–Burk plot of (a) free PGA and (b) immobilized PGA. Synthesisconditions:8.50wt% Fe3O4, 60% MBAA, and $W_{(GMA)}$ /$W_{(NVP)}$=1:1[117] (Ping Xue et al. Copyright 2015, Elsevier)

Ping Xue[117] et al. prepared magnetic hydrophilic polymeric microspheres containing oxirane groups by inverse suspension polymerization of glycidyl methacrylate (GMA), N, N'-methylene bisacrylamide (MBAA) and N-vinyl pyrrolidone (NVP) in the existence of formamide, which were denoted as magnetic poly(GMA-MBAA-NVP) microspheres. The magnetic poly(GMA-MBAA-NVP) microspheres were characterized by scanning electron microscopy (SEM) (which was shown in **Figure.7**(a)(b)(c)), FT-IR spectroscopy, X-ray diffraction (XRD), vibrating sample magnetometer (VSM) and so on. The results showed that poly(GMA-MBAA-NVP) microspheres possessed significant spherical shape, narrow size distribution, abundant porous structure, reactive oxirane groups and superparamagnetic properties. However, as can be seen from the results of free PGA and immobilized

PGA hydrolysis rate, the affinity of immobilized PGA to the substrate is lower than that of free PGA, which leads to the increase of the concentration of substrate at the maximum reaction rate. It is proposed that the diffusion of substrate in magnetic polymer microspheres is limited after the enzyme immobilization. Although the magnetic microspheres are hydrophilic, the pore structure is still bad for the diffusion of substrate, which results in the decrease of the maximum mass transfer rate for immobilized PGA.

3.2.5. New Carrier

Based on the above research results of inorganic, organic and composite carrier, Although the inorganic carrier has good operational stability, it cannot effectively balance the relationship among the loading amount, the response rate and the carrier recovery rate, which can increase the difficulty of recovery. For organic, carriers, although they are easy to modify, but the large molecular weight always caused a low response rate. Magnetic nano-composites containing the advantages of inorganic and organic carriers can easily recover the immobilized PGA by using magnetic fields, however, magnetic nano-particles are easily oxidized by air and are not durable in carrier recovery (Some immobilized vectors and immobilized methods are shown in **Table 1**). Therefore, developing and designing new carrier types is still urgent.

Temperature-sensitive polymers are temperature-responsive materials, its conformation can undergo reversible changes between the sol and the gel as a function of ambient temperature[149-152]. The temperature-sensitive polymers with low critical phase transition temperatures (LCST) are an important type of temperature sensitive polymers. The LCST polymer can form a hydrogen bond with water molecules in a water medium because it contains hydrophilic groups such as a substituted amide or a hydroxyl group in the molecule[149, 153]. Moreover, as the temperature changes, the ratio of hydrophilicity to hydrophobicity in polymer will change significantly, which in turn leads to the mutual

conversion of the sol and the gel. Due to the unique molecular structure and functional group characteristics of LCST polymer, researchers can effectively use the change of ambient temperature to control the phase behavior of materials, and then realize the intelligentization of materials. Now LCST polymer has been applied to biomedical[154, 155], catalytic[156, 157], sensor[158-160], immobilized enzymes[161-163] and other fields. In the field of immobilized enzymes, Ke Li et al. [112] synthesized the tri-block temperature-sensitive polymer PDEA-b-PHEMA-b-PGMA and applied it to the PGA immobilization carrier for the first time, successfully using the polymer sol state realize the immobilization of the PGA and the catalytic conversion of the immobilized PGA, which effectively increased the mass transfer rate, and the recovery of the immobilized PGA in the gel state (**Figure.8**). However, the results showed that although the carrier type increased the immobilized PGA mass transfer rate, its recovery performance was still lower than that of magnetic nanocarriers. Based on the characteristics of the above temperature-sensitive polymers and magnetic materials, if the composite carrier with magnetic-temperature sensitivity can be designed and synthesized, the temperature-sensitive property can be used to improve the response rate of the immobilized PGA, and the recovery of the carrier can be easily realized by using the magnetic properties. The new smart carrier will promote the development of immobilized PGA.

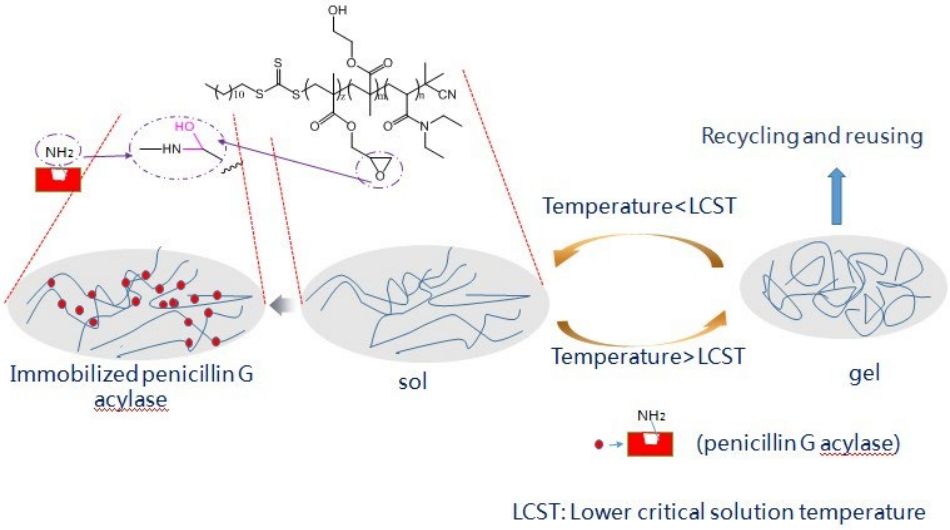


Figure.8 Schematic representation of thermo-sensitive polymer immobilized PGA. When the ambient temperature was lower than the PDEA-b-PHEMA-b-PGMA's LCST, the thermo-sensitive polymer presented a sol state, which was conducive to the immobilization of PGA and catalytic transformation of the immobilized PGA. When the ambient temperature is higher than the PDEA-b-PHEMA-b-PGMA's LCST, the temperature sensitive polymer is in gel state, which is conducive to the recovery and reuse of the carrier.

3.2.6. The Problem of the Carrier

Organic synthetic polymer carriers and magnetic nanocomposite are an important research target for immobilized carriers, although the response rate and reusability of immobilized PGA has been improved to varying degrees, but the progress on immobilized PGA activity recovery rates has been slower. Researchers analyzed that the main reason for the low activity recovery rate of immobilized PGA is that the suitability of the carrier microenvironment and PGA isn't strong, ZD Knežević-Jugović[108], P. Xue[117] and AI Kallenberg[164] et al. found that when the density of the immobilized targets is large, the multi-point fixation of immobilized PGA is an important reason for the low activity recovery rate of immobilized PGA (Because the molecular size of PGA is relatively large relative to the target, and the active center of PGA is composed of weak interaction forces such as hydrogen bonding, hydrophobic interaction, et al, which is easily deformed by the influence of microenvironment (temperature, pH, target density, etc.). When the target density is too large, because there are more types and quantities of functional groups available for reaction on the PGA, as a result, a PGA can be immobilized by multiple targets, which will cause the conformation of the PGA active center to be deformed, so the activity is lowered. However, combining the research work of our laboratory on immobilized PGA[112], in addition to the above inference, here is a new conjecture: The mutual extrusion may lead to distortion of the conformation of the PGA activity center under stress, which leads to a decrease in enzyme activity recovery. However, the current research has not proved the essence of the influence of target density

on the enzyme recovery rate, therefore, taking corresponding measures to improve this condition and further improve the enzyme activity of immobilized PGA should arouse the attention of researchers.

In addition to the target density of the carrier described above it has an important effect on the activity of the immobilized PGA, the hydrophilic/hydrophobicity of the carrier is also an important factor affecting the activity of immobilized PGA, it can be attributed to the structure and catalytic mechanism of PGA, because PGA is a hydrolase, increasing the hydrophilicity of the carrier is beneficial to the immobilization of PGA. In addition, PGA requires a certain amount of water molecules to form its active center conformation[165]. Therefore, the hydrophilic carrier is advantageous for increasing the probability of contact of the immobilized PGA with substrate, thereby improving the catalytic performance. According to the reports, hydrophilic monomers are often used to construct the hydrophilicity of the carrier, such as acrylamide-based polymers, N-vinylpyrrolidone, β-hydroxyethyl methacrylate and et al[117, 166]. However, since the conformation of the PGA active center is composed of weak interactions such as hydrogen bonding and hydrophobic interaction, there are many factors affecting the activity of the PGA enzyme, not only by the hydrophilic/hydrophobicity of the monomer but also by the monomer polarity, etc. Although researchers have investigated the effect of organic solvents (such as normal paraffins, polychloromethanes, halogenated alkanes, ethers, monohydric alcohols, polyols, monobasic acids, polybasic acids, etc.) on the enzyme activity of free PGA[167]. However, due to the design of the immobilized PGA carrier is to succumb to the performance of the immobilized PGA, and different polymerization methods are selected depending on the kind of the carrier. According to the current polymerization method (For example, the reversible addition-fragmentation chain transfer (RAFT) method, atomic transfer radical polymerization (ATRP) method and so on), which are not suitable for all monomers, it is necessary to select the monomer type according to the polymerization method. Therefore, the influence of the organic solvents on the activity of free PGA could not provide theoretical support for the selection of monomers in the polymer carrier. However,

according to the current research progress of carrier monomer selection, it can be found there is a lack of systematic studies of functional monomers on the immobilized PGA activity. If the study of functional monomers on the immobilized PGA activity can be systematically studied and can obtain the rules, which will have important guiding significance for further improving the performance of immobilized PGA.

3.3. Reaction Medium - Two Phase System

In the process of PGA catalyzed PG, in addition to the formation of 6-APA, along with the formation of this by-product phenyl acetic acid, it is a competitive inhibitor of PGA[168]. In the reaction system, when the concentration of phenyl acetic acid is large, it will inhibit the catalytic efficiency of PGA[146, 169]. In the actual production process, effectively separating the immobilized PGA from the reaction product and the substrate can not only improve the catalytic efficiency of the PGA, but also improve the yield of 6-APA. Traditional industries use two-phase water technology to achieve the separation of immobilized PGA, substrates and products[170]. Two-phase water technology is that two water-soluble polymers or a high polymer and a salt can form two layers of incompatible aqueous solution. The principle of two-phase material distribution is similar to that of water organic solvent extraction and is essentially based on the selective distribution of biomolecules in two-aqueous systems. The distribution of matter between two phases is determined by some interaction between the partitioned material and the phase-forming polymer or salt, such as intermolecular forces, charge forces, etc., but also depends on some environmental factors. Under the influence of these factors, the substance enters the two aqueous phase system and is selectively distributed to one of the phases, thereby achieving the effect of material separation[171-173].

Cao Xuejun et al. [174] first studied the kinetics of PGA derived from E.coli ATCC11105 in two aqueous systems, and conducted basic research on the catalytic production of 6-APA in the two aqueous

phases. Subsequently, they studied the separation of PGA derived from immobilized cells in the two phases, which achieved the immobilized cells to be completely distributed in the lower phase, but the cells occupied a larger volume and the products tend to be evenly distributed. Andersson et al.[175] used free PGA to break down PG into 6-APA in two aqueous phases and obtained that the stability of free PGA was poor and unilateral distribution was difficult. Terreni et al. [176] used a two-phase system to promote the hydrolysis of PGA. Results showed the acylation product had a phase partition coefficient greater than 200, and the kinetic yield and thermodynamic yield were greatly improved. Although the above two-phase system can effectively increase the yield of PGA, but it had several disadvantages such as the phase-forming substance is expensive, cannot be recycled, pollutes the environment and so on.

In order to solve these problems, the new reusable two-phase system is applied to the enzyme-catalyzed reaction[177, 178]. There are many different types, including light-sensitive systems, sensitive systems, pressure-sensitive systems, and magnetic sensitive systems, depending on the requirements of the reaction and separation[179]. The phase-forming polymers of these two aqueous phase systems can be varied in response to certain specific conditions which can be recycled for reuse. Jin Keming et al.[180] used a two-phase system consisting of a light-sensitive recyclable polymer to cleave PG using immobilized PGA, the laser can be recovered by laser irradiation and conditioning, which is a better improvement than the previous reaction system, the reaction yield under this condition is increased by nearly 20% compared to the aqueous phase reaction under similar conditions.

However, the industrial application of the two aqueous phases is still in the early stage of research, and there is no large-scale production. Because the partition coefficient of immobilized PGA and substrate and product in two phases is affected by many factors, such as pH, polymer concentration, polymer molecular weight, system temperature, inorganic salt ions, etc., which increases the difficulty of research. At present, how to realize the equilibrium of phase system, selectivity and product distribution

is the problem of two-phase technology. In addition, this technology also has the following problems in large-scale application processes, such as: first, it needs to study the conditions of its amplification and continuous extraction operations, which can be combined with a continuous separation device for separating biological products. Second, how to process steps and dispense time to avoid defects in sample loss and prolonged analysis time caused by batch two aqueous phase system. In summary, the two aqueous phase system has broad development prospects in the PGA catalytic reaction.

3.4. Regeneration of Immobilized PGA

Studies on immobilized PGA regeneration were first derived from immobilized PGA synthesis of cephalexin, because during the actual production of immobilized PGA in the synthesis of cephalexin, when the immobilized PGA activity was reduced to 50% of the initial activity, it becomes a waste enzyme and cannot be used any more. Therefore, this causes a large amount of waste enzyme to be produced in industrial production[181]. Considering the catalytic efficiency and economic cost, if these waste immobilized PGA can be restored to activity and the use of the batch is extended. If the study of immobilized PGA regeneration is used to catalyze the reaction of PG to generate 6-APA, it also has important practical application value. The reason for the inactivation of the immobilized PGA is the conformational change of their three-dimensional structure. Partial or total recovery of enzyme activity can be achieved if the appropriate configuration is to reverse the conformation of the immobilized PGA toward the original form. Miranda[182] has done research on immobilized PGA regeneration, firstly, the study on the regeneration of immobilized PGA found that even highly stable enzyme preparations may be inactivated for a long time in the presence of organic co-solvents or high temperatures, thus reducing the operational life of the biocatalyst. In the latter research process, Miranda et al. [183] also carried out two strategies for the regeneration of immobilized PGA, one was to expand in an aqueous medium-refolding increased the enzyme activity from 36.0% to 62.8%, and the other was through the addition of

30.0% ethylene glycol (EG) in the aqueous medium to activate PGA (The conformation of the active center is restored) increased the enzyme activity from 50.0% to 68.4%. In addition, it was reported that using a noncompetitive inhibitor (7-ADCA and 6-APA) in the co-solvent as a medium, the PGA activity can be increased from 36% to 96% by the unfolding-refolding strategy, but PGA activity increased from 50.0% to 98% by direct regeneration. Romero et al. [184] studied the regeneration of immobilized PGA from three immobilized carriers: PGA immobilized by covalent attachment to cyanogen bromide Sepharose (CNBr Sep-PGA); the other two were multiple sites that were covalently attached to immobilized PGA with different adhesion strengths of glyoxylose agarose (Gx1-PGA and Gx2-PGA). The above three immobilized PGAs were inactivated with biocatalyst 70% (v/v) dioxane at 5^{o}C to the remaining 25% activity. The activity of Gx1-PGA and Gx2-PGA was restored by 70% using the unfolding-refolding strategy, but CNBr Sep-PGA was completely inactivated after unfolding and could not be recovered. Soothinget al. [181] studied the effects of detergent, ultrasound, medium, etc. on the regeneration of inactivated immobilized PGA, results showed that deionized water washing and acid water (pH=6) washing had no regeneration effect on inactivated immobilized PGA, but alkaline water (pH=8) washing has a regenerative effect. After washing with pH=8 alkaline water for 60 min at 30^{o}C, the activity of immobilized PGA was increased by 5.7%. Sonarization (frequency 28 KHz, 15 min) increased the inactivation of immobilized PGA activity by 5.67%. The expansion-refolding regeneration method is ineffective for regeneration of inactivated immobilized PGA. The medium direct regeneration method (30% ethylene glycol + 100 Mm 7-ADCA) has a regeneration effect on inactivated immobilized PGA, which increases the enzyme activity by 13.81%. The suitable regeneration method for inactivation of immobilized PGA is as follows: firstly, soak for 30 min with 30% ethylene glycol + 100 mM 7-ADCA solution, then wash with water for 60 min, and finally sonicate with 28 KHz for 15 min to inactivate the immobilized PGA enzyme. The recovery rate is 77.75%, and the regeneration effect is good.

In addition to the above studies on the effect of immobilized PGA regeneration, if to explore the effect of dimethyl sulfoxide (DMSO) on the regeneration of immobilized PGA may be a good way. It has been reported that Yi Chen et al. [185] studied the role of DMSO to activity tyrosinase II (TYR) by enzyme kinetics in a buffer system of 30^{o}C, pH= 6.8 Na_2HPO_4-NaH_2PO, results showed that when the integral value of DMSO solution was ⩽25 %, the activity of TYR increased correspondingly as the fraction of dissolved liquid in DMSO increased. Ping He et al.[186] used UV absorption spectroscopy and fluorescence emission spectroscopy to study the effect of dimethyl sulfoxide on papain activity and conformation, results showed that low volume fraction of DMSO had little effect on the conformation of papain. Moreover, DMSO also exhibits the second effect on chitinase, papain, and other enzymes, researchers explored the interaction of DMSO with enzymes and concluded that the conformation of the enzyme is maintained by the secondary bonds in the enzyme molecule, especially the hydrogen bond and the hydrophobic bond, and the active site of the enzyme has a certain flexibility, when the reaction system contains an appropriate amount of DMSO, it may affect the interaction between the side chain of the enzyme molecule or between the enzyme molecule and water, resulting in slight changes in the force of maintaining the conformation of the protein molecule, which may increase the flexibility of the active site of the enzyme. Since PGA is also a protein in nature, and its active living center conformation is also composed of hydrogen bonding, hydrophobic interaction, etc., if it can be inferred that the addition of an appropriate amount of DMSO may also be able to effectively increase the activity of PGA. It is necessary to investigate the effect of DMSO on the regeneration of immobilized PGA.

At present, there are few studies on the regeneration of immobilized PGA, and the research on immobilized PGA regeneration is still in the inquiry stage. Once the research on the regeneration of the immobilized PGA have well results, it is not only theoretically significant, but will more so make a

significant contribution to saving the economic cost of the enterprise and improving its economic efficiency.

3.5. Reactor

In the process of producing 6-APA by immobilized PGA, the enzyme cleavage reaction is a key step, and how to design a suitable reactor is of great significance to improve the reaction efficiency, simplify the subsequent separation operation, improve the yield and quality of 6-APA and reduce the overall production cost[187]. Currently, studies on immobilized PGA reactors include the following: stirred tank reactors, column packed bed reactors, enzyme membrane reactor, electro dialysis coupling reactor and isoelectric immobilized enzyme reactor.

3.5.1. Stirred Tank Reactor

Stirred tank reactor is widely used in industrial production of 6-APA. Stirred tank reactor is divided into batch stirred tank reactor (BSTR) and continuous flow stirred tank reactor (CSTR)(Their brief structures are (a) and (b) as shown in **Figure 9**). The above two stirred reactors have the advantages of adjustable temperature and pH of reaction liquid, sufficient stirring, low diffusion resistance, fast reaction speed, high efficiency and so on[187, 188]. Fonseca et al. [189] studied the enzymatic preparation of 6-APA in a stirred reactor by batch operation, results showed the yield of 6-APA reached 93% at the concentration of PG 8% (w/v), pH=8, and 35^{o}C. Ospina et al. [190]also studied the conversion process of 6-APA under batch operation, results showed the yield of 6-APA reached 93% at the concentration of PG 12% (w/v), pH=7.5 and 37^{o}C (differences in the optimal catalytic conditions for the above two 6-APAs may result in different properties of the carrier). However, the above two types of reactor also have differences, for example, BSTR reactor needs to recover immobilized PGA by centrifugation or filtration precipitation, because of the strong force in the recovery process, it is easy to cause loss of enzyme activity. Compared with BSTR, CSTR has more advantages in the recovery of

immobilized PGA, it just to install filters at the outlet of the reactor, or immobilizing PGA with nylon mesh and then installing them on the stirring shaft, or by using synthetic magnetic nanocomposites as immobilized carriers and recovered it by magnetic field. The continuous production of immobilized PGA can be realized by immobilizing the PGA on the vessel wall or on the stirring shaft. Although stirred tank reactor has the above advantages, intense stirring still causes mechanical damage to immobilized PGA, and the excessive local alkali concentration caused by adjusting the pH value of the solution with alkali can cause the inactivation of immobilized PGA, Therefore, the reactor still needs further improvement.

3.5.2. Column Packed Bed Reactor

Column packed bed reactor is a kind of reactor which mixes immobilized PGA with diatomite, perlite or fiber powder, and then fills the thin bed, PG solution is stored in a stirred tank[191]. Column packed bed reactor can be divided into fixed bed reactor and fluid bed reactor(Their brief structures are (c) and (d) as shown in **Figure 9**). These two reactors have the following advantages: separating the reaction solution from immobilized PGA can effectively avoid the damage of immobilized enzyme structure caused by stirring in stirred tank reactor, besides, avoiding the contact between immobilized PGA and alkali solution, and reduce the loss of immobilized PGA activity caused by excessive local pH. The reactor prolonged the life of immobilized PGA and increased its recycling batches, thus reducing the production cost of 6-APA[192]. Because the two reactors also have some differences, therefore, the fixed bed reactor and fluid bed reactor are introduced respectively, and their problems are briefly analyzed.

Fixed bed reactor is suitable for immobilized PGA with larger particles[193]. Because the catalyst is centralized and stored together, the filling density of the catalyst is high, the relative effective volume ratio of the reactor is large, therefore, the volume productivity per unit time is higher than other types of reactor. In addition, the residence time distribution of fluids in different parts of the reactor can be

controlled in a very narrow range, resulting in a concentration gradient, high concentration at the inlet of the substrate, low concentration at the outlet, and almost all of the substrates can be converted into products. Most importantly, because the reactor can separate the immobilized PGA from the reaction substrate and products, it can effectively solve the influence of phenyl acetic acid (by-product) inhibitors on the catalytic performance of immobilized PGA, thereby improving mass transfer rate. However, the immobilized bed also has the following disadvantages: temperature and pH are difficult to control; substrate and products will produce axial concentration distribution; cleaning and replacing part of the immobilized enzyme is more troublesome, *etc*[194].

Different from fixed bed reactor PGA, the particles of immobilized PGA in fluid bed reactor are loose[195]. The substrate enters the reactor from the bottom and flows upward through the bed, in the bed, the density of particles is greater than that of the substrate. Besides, the bottom flow makes the immobilized PGA particles fluidized in the bed. Fixed bed is suitable for the conditions of low viscosity of medium and lower velocity of flow than entrainment velocity of particles. In order to reduce particle spillover, the upper part of the reactor is usually designed as a conical enlargement to reduce the flow rate. The advantages of the reactor are as follows: good mass and heat transfer performance. The pH, temperature control and gas supply are relatively easy. It is not easy to plug, and can be used to treat liquid with high viscosity. Powdery substrates can be treated without high pressure drop even if fine particles are used as catalysts. However, it also has the following shortcomings: Because of the large void volume of fixed bed, the concentration of immobilized PGA is not high, therefore, volume productivity per unit time is low in fixed bed reactor. Because of the high-speed flow of substrate, immobilized PGA could be washed out, which leads to the loss of immobilized PGA and reduces the conversion rate. There are usually two ways to avoid enzyme loss, one is to circulate the substrate, which can avoid the catalyst out, so that the substrate completely into the product; another method is to use a reactor group consisting of several fluidized beds or a conical fluidized bed[50, 196].

At present, multi-stage continuous column reactors (generally 2 to 6 columns) have been industrialized, and the pH can be adjusted between the packed columns, which greatly simplifies the operation steps[50]. However, the filler in the column packed bed reactor is a powder such as diatomaceous earth, perlite or fiber powder, which increases the diffusion resistance of the immobilized PGA and its substrate and product, and the reaction efficiency is lower than that of the stirred tank reactor. Because it affects the catalytic efficiency of the immobilized PGA, and is prone to severe bed pH grading, further reducing the enzyme activity of the immobilized PGA, therefore, it is generally suitable to use a series of column packed bed combination reactors when cracking a low concentration of PG in industrial production; a higher concentration of PG is used, and a stirred tank reactor is suitably used.

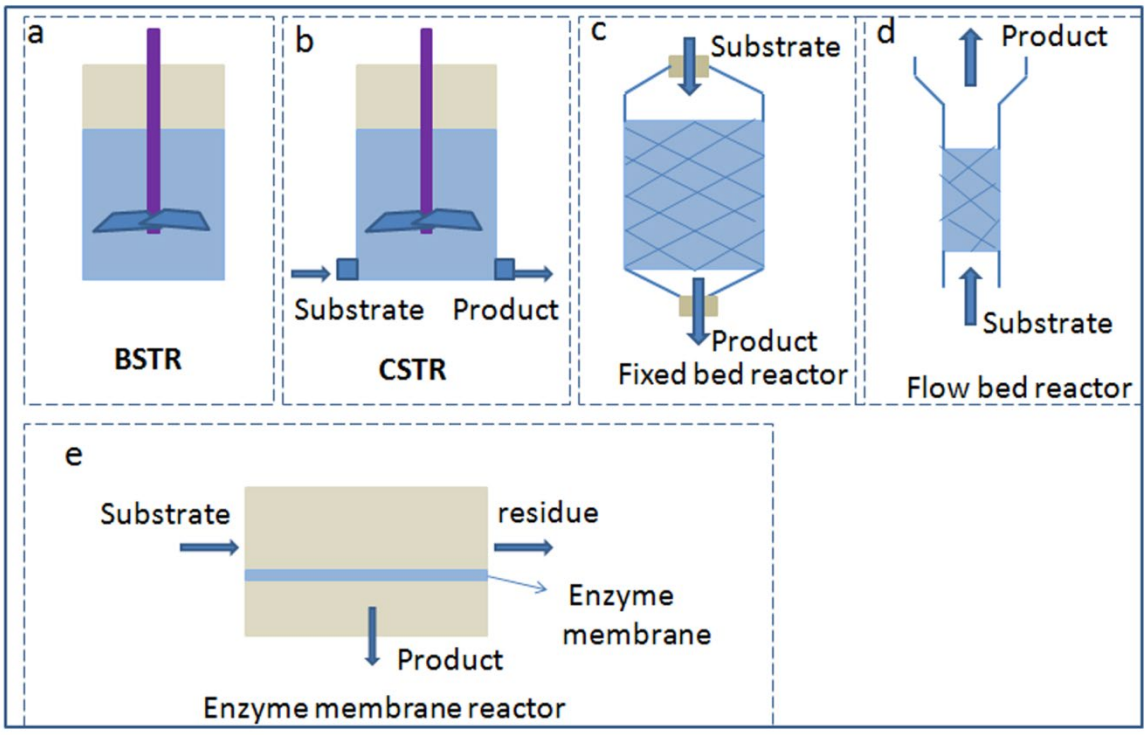


Figure.9 Brief structure of the reactor. (a) Batch stirred tank reactor; (b) Continuous flow stirred tank reactor ;(c) Fixed bed reactor ;(d) Fluid bed reactor; (e) Enzyme membrane reactor

3.5.3. Enzyme membrane reactor

The enzyme membrane reactor is a novel membrane reactor that is based on a selective semipermeable membrane to separate enzymes and products (or substrates) and is currently in the industrial application stage[197](It structure is (e) as shown in **Figure 9**). It integrates product separation, purification and immobilized PGA recovery and utilization, and has advantages unmatched by other methods[198, 199]. The high specific surface area of the membrane greatly improves the catalytic efficiency of immobilized PGA, and can effectively separate the product, thereby reducing the inhibition of immobilized PGA by the excessive concentration of phenyl acetic acid. Depending on the type of membrane module, membrane type reactors can be divided into plate and frame, spiral wound, tubular and hollow fiber type membrane reactors. Due to the comprehensive consideration of the structural complexity, packing density, membrane replacement, anti-pollution ability, cleaning, liquid demand, cost, etc. of the above membrane type reactor, hollow fiber membrane reactor has a large specific surface area and reacts, its structure and operation of the device are flexible and changeable, and it is a membrane type reactor which is currently researched more deeply.

At present, hollow fiber membrane reactors used in industry are mainly flow-controlled hollow fiber membrane reactors, which overcome the disadvantages of low diffusion mass transfer rate of diffusion-controlled hollow fiber membrane reactors[200, 201]. Hollow fiber membrane reactors can be divided into three types: ultrafiltration flow-controlled hollow fiber membrane reactor, circulation flow-controlled hollow fiber membrane reactor and recoil flow-controlled hollow fiber membrane reactor[50]. Among them, because circulation flow-controlled hollow fiber membrane reactor is not suitable for large-scale production applications, ultrafiltration hollow fiber membrane reactors and recoil hollow fiber membrane reactors have great application prospects. Ultrafiltration hollow fiber membrane reactor is essentially a combination of a continuous stirred tank and an ultrafilter[202], because it combines the advantages of a membrane reactor and a stirred reactor, immobilized PGA is circulated at

a high speed in the system, substrate is continuously fed at the bottom end and product is continuously discharged from the top of the reactor, which not only increases the catalytic reaction area, but also increases the catalytic efficiency. However, since immobilized PGA circulates at high speed in the system and the shear stress is large, it is easy to cause immobilized PGA to be broken and deactivated. In addition, in the process of operation, in order to achieve a high conversion rate, the continuous flow acceleration rate of substrate needs to be controlled at a low speed, which increases the difficulty of operation. Therefore, at present, ultrafiltration membrane hollow fiber membrane reactor is still in the research stage.

In the recoil type hollow fiber membrane reactor, PGA is trapped on one side of the membrane, and the material is forced to react with the catalyst by pressure and then flows out of the membrane from the other side. It has the same advantages that immobilized PGA has such as high specific surface area, easy immobilization and easy operation, therefore, immobilized PGA has a large transfer rate and catalysis. However, due to the accumulation of immobilized PGA on the membrane surface, it is easy to cause the leakage of immobilized PGA and the serious decrease of the permeability of hollow fiber membranes, which will affect the stable operation of the reactor[203]. In addition, the reactor requires high permeability flux for hollow fiber membranes and high operating pressure, which is still a bottleneck problem in reactor design.

Because PGA is immobilized on the membrane for catalytic conversion, the nature of the immobilized membrane directly affects its catalytic efficiency and yield. Although the above membrane type reactors have different advantages and disadvantages, they still have some common problems to be improved for the immobilized PGA membrane: First, due to the steric hindrance effect during immobilization of PGA, which allows the immobilized PGA to be inactivated due to changes in spatial conformation or obscuration of the active site[204]. Therefore, it is necessary to replace the random immobilization with directional immobilization to ensure that the spatial configuration of PGA remains

unchanged. In addition, the side chain groups of PGA can be subjected to the necessary chemical modification to increase the biocompatibility between PGA and the membrane material and the tolerance of PGA to organic solvent, and at the same time, the molecular weight of immobilized PGA should be increased to improve the entrapment effect of the membrane. Because the current membrane processing level is low, the membrane aperture distribution and the morphology structure uniformity are poor, which easily causes leakage of immobilized PGA. Besides, the introduction of organic solvents often causes the film to swell, which causes the film to bend and deform, and loses the effective mass transfer area. Therefore, it is necessary to develop a film material resistant to swelling, and develop a polymer membrane material with good biocompatibility and anti-pollution. In addition, advanced film forming technology should be adopted to optimize the film shape and structure.

3.5.4. Electro dialysis coupling reactor

Electro dialysis coupling reactor is mainly a competitive adsorption reaction on the negative ion exchange membrane, which has a device with superior advantages in enzyme separation[205]. It has the advantages of simple operation, short separation time and high separation efficiency. In the electro dialysis coupling reactor, substrate(it is PG) and product (they are is phenyl acetic acid and 6-APA) exist in the form of monovalent negative ions, since the mass transfer rate is different on the negative ion membrane, the separation can be effectively achieved by using the negative ion exchange membrane[50]. However, due to the different mass transfer rates of the substances, it is easy to cause concentration polarization on the ion exchange membrane, which not only affects the catalytic efficiency, but also increases the operation difficulty, which is the main problem electro dialysis coupling reactor faces, therefore, electro dialysis coupling reactor is still in the research stage.

3.5.5. Isoelectric immobilized enzyme reactor

Isoelectric immobilized enzyme reactor uses the electromechanical principle to trap and fix the PGA in an electrolysis chamber surrounded by an isotropic isoelectric film of isoelectric points 5.5 and 9.0 with using batch feed, accompanied by short-term interruption of the electric field[206]. In the double discontinuous mode of operation, the target product 6-APA is trapped in an electrolysis chamber surrounded by an amphoteric membrane of isoelectric points 3.5 and 5.5 due to its amphoteric characteristics; the by-product phenyl acetic acid is initially accumulated in this chamber, later, it gradually enters the anode storage chamber; unreacted PG rarely leaks out of the reaction chamber into the anode region to contaminate the product[50]. Therefore, the isoelectric immobilized enzyme reactor separates the reaction by-products while completing the reaction, simplifying the separation step in the subsequent 6-APA preparation process. However, at present, there is also a concentration polarization phenomenon in this type of reactor, which needs further study.

4. CONCLUSION AND PERSPECTIVES

In the review, combining the performance of immobilized PGA, such as activity recovery, mass transfer rate, reusability, recyclability, etc., the requirements for immobilization methods, carriers and two-phase media, development status, advantages/disadvantages, problems and the solution is summarized. Besides, according to the nature of PGA, the causes of defects such as immobilization method, carrier, two-phase medium, immobilized PGA regeneration and reactor are analyzed. As for the immobilization methods, good results have been obtained at present, and GMA containing epoxy groups is generally used as the immobilization target. Because the immobilized carrier is the main factor affecting the performance of the immobilized PGA, the microenvironment of the carrier (structure, composition, target density, monomer properties, etc.) has an important influence on the performance of the immobilized PGA. In order to solve the problem of compatibility of immobilized carrier in terms of

activity recovery, mass transfer rate, reusability, recyclability, etc., the polymers with carrier magnetic and temperature-sensitive was proposed to provide ideas for solving the above problems. In addition, for the phenomenon that the immobilized PGA activity is low because the immobilized target density is too high, researchers generally believe that the immobilized PGA multipoint fixed the cause of the above phenomenon, however, combined with relevant research experience, the author puts forward extrusion between immobilized PGA may also be a major cause of the enzyme activity recovery rate decline, however, about the above two kinds of inference, there is no experiments to verify. In addition, the hydrophilic/hydrophobic properties of the monomer in the carrier have an important influence on the activity recovery of the immobilized PGA. However, in the design process of the carrier, the selection of monomer has a lack of rules to be abided by. Therefore, it is necessary to systematically study the influence of the properties of functional monomer on the enzyme activity of the immobilized PGA. DMSO was first proposed as a reference solution to improve the regeneration of immobilized PGA activity. Because, the traditional reaction medium - two - phase system exists the phase-forming substance is expensive, cannot be recycled, pollutes the environment and so on. Therefore, the new reusable two-phase system is applied to the enzyme-catalyzed reaction. Because it effectively avoids the above disadvantages, it has potential research value and market space. The reactor plays an important role in catalytic efficiency and yield of immobilized PGA. If membrane reactor can be combined with electrodialysis technology, it will be of great significance to save reaction time and improve yield, etc., however, there are currently technical limitations.

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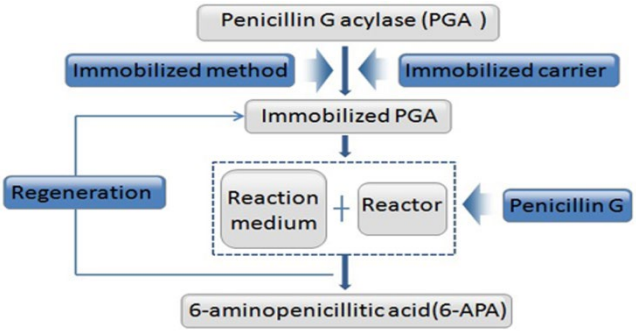
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