秋田県立大学大学院博士学位論文

Preparation of Functional Porous Biochar for Cellulase Immobilization

機能性多孔質バイオチャーの創製および固定化担体への応用に関する研究

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Abstract

In recent years, new sustainable energy, especially bioethanol, has received wide attention. As a “green” approach, the preparation of bioethanol from lignocellulosic biomass by enzymatic hydrolysis and bio-fermentation is very popular, due to the environmentally friendly and low production cost. Cellulase is an important role in the biodegradation of cellulose to glucose. Because the enzymatic hydrolysis could be effectively and specifically conducted under mild conditions. However, some factors limit the application of free cellulases, such as changes in pH, temperature and ionic strength, product inhibition, and difficulty in recovering from the reaction medium. Therefore, it is meaningful to improve the stability and reusability of cellulase. The use of immobilization technology can improve the stability of the enzyme and enable it to be recycled and reused. The key point of this technology is the choice of the immobilization method and support. In this study, porous biochar (obtained from bagasse) combined with chitosan and magnetic particles to prepare several functional carriers for cellulase immobilization via covalent bonding or adsorption. The effects of chemical characteristics and morphology of the support on the cellulase activity recover and recycle were discussed.

In chapter 1, the research background and the construction of this thesis are described. The objectives of the research are to study the preparation of functional porous biochar and its application for cellulase.

In chapter 2, the properties of materials used in this thesis, experimental methods, and characterization are presented.

In chapter 3, porous biochar derived from sugarcane bagasse was prepared and then coated with different amounts of chitosan (C@CS) for cellulase immobilization. Cellulase was covalently immobilized on the support by using glutaraldehyde as a linker. The chemical characteristics and morphology of the samples were determined by SEM, BET, FT-IR and XPS. The properties of immobilized enzyme were evaluated

by activity recovery, optimum pH value and temperature, and recyclability. The results showed that all the three kinds of immobilized cellulase did not change the optimum pH value of 4 and temperature of 60 °C, and they also exhibited good activity and reusability. Especially for C@CS25 (the feeding ratio of porous biochar to chitosan was 0.5 g : 25 mg), the support retained the morphology of porous biochar well. The corresponding immobilized cellulase kept 67% activity of free cellulase at pH = 4 and 60 °C, and showed a glucose productivity of 90.8% even after 10 cycles.

In chapter 4, because of the high specific surface area, polyporous structure and ease of preparation, porous biochar from lignocellulosic biomass is popular for being used as support for enzyme immobilization. In this work, polyporous biochar combined with magnetic particle γ-Fe_2O_3 was prepared by calcination and then used as support for cellulase adsorption. The effects of calcination temperature and time on the properties of magnetic polyporous biochar were investigated and the optimum preparation condition was obtained. For the cellulase adsorption, the immobilization capacity for the magnetic support reached as high as 266 mg/g with a relative activity of 73.6% compared with free cellulase. The behavior of cellulase adsorption showed that an endothermic process occurred more easily at high temperatures, which resulted in a high adsorption amount.

In chapter 5, the porous biochar was obtained from sugarcane bagasse by alkali activation and pyrolysis and then magnetized with γ-Fe_2O_3 by calcination. After functionalization with chitosan and activation with glutaraldehyde, the as-prepared chitosan/magnetic porous biochar was served as support to immobilize cellulase by the covalent bonds. The immobilization amount of cellulase was 80.5 mg cellulase/g support at pH=5 and 25 ºC for 12 h immobilization. To determine the enzymatic properties, 1% CMC (dissolved in 0.1 M buffer.) has been considered as a substrate for hydrolysis at different pH values (from 3 to 7) and temperatures (30 to 70 ºC) for 30 min. The results have shown that the optimum pH and temperature of the free and immobilized cellulase did not change, which was pH 4 and 60 ºC, respectively. The immobilized cellulase has a relatively high activity recovery of 73.0%. However, it also exhibited a higher Km value and a slower Vmax value comparing to the free enzyme. In the reusability assay, the immobilized cellulase showed initial glucose productivity

of 330.9 mg glucose/g CMC and kept 86.0% after 10 uses. In conclusion, the chitosan/magnetic porous biochar has great potential applications as support for enzyme immobilization.

In chapter 6, general conclusions of the study are made.

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Chapter 1 Introduction

1.1 Background

In recent years, new sustainable energy, especially bioethanol, has received wide attention. As a “green” approach, it is popular to prepare bioethanol from lignocellulosic biomass by enzymatic hydrolysis and bio-fermentation, due to the environmentally friendly and low production cost [1–3]. Cellulose and hemicellulose could be hydrolyzed to reducing sugars, then the sugars are fermented into ethanol. For the process of hydrolyzing lignocellulosic materials, the use of strong acids or alkalis could increase the burden on the environment and equipment, but enzymatic hydrolysis would not. Therefore, enzymatic hydrolysis of lignocellulosic should be a greenway to produce fermentable reducing sugars [4–6].

Cellulase, a composite enzyme, mainly compose by endo-l, 4-β-D-glucanase, exo-l, 4-β-D-glucanase and β-glucosidase. Its classification is based on attacking the depolymerization stage of the substrate. Endoglucanases randomly hydrolyze the glycosidic bonds in the amorphous regions of cellulose to produce oligomers with several degrees of polymerization. Then, exoglucanase hydrolyzes the β-1, 4-glycosidic bond of the oligomer to produce cellobiose. Finally, cellobiose is degraded to glucose by β-glucosidase [7]. However, there are limitations in the practical application of cellulase, such as unstable, difficult to recover and reuse during the hydrolysis process [8,9]. In facts, there are several methods could be used for enhancing the stability of the enzyme, such as protein engineering, chemical modification, and immobilization [10,11]. Among them, immobilization has more advantages in the isomerization of the enzymatic reactions and reusability [12]. Thus, the cellulase immobilization is required for easy recovering and reusing.

1.2 Methods of enzyme immobilization

Basically, the immobilization of enzyme is a method by which a protein is bound to a solid support through physical or chemical methods to eliminate diffusion of the protein in the hydrolysis process. And the methods can be divided into binding to a support, entrapment, and crosslink [13]. Fig. 1-1. illustrates the basic methods of enzyme immobilization [14].

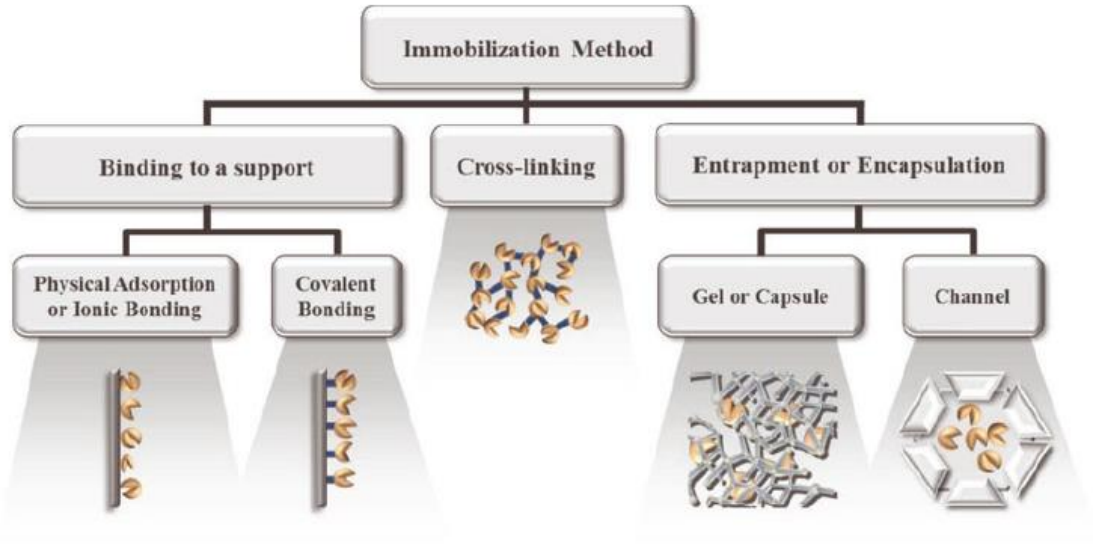

Fig. 1-1. Overview of the different enzyme immobilization methods [14].

1.2.1 Binding to a support

Binding to a support refers to the immobilization of the enzyme on a water-insoluble support. These methods can be divided into physical adsorption, ion binding, and covalent bonding according to the physical and chemical properties of enzymes and supports [15].

(1) Physical adsorption

Physical adsorption is simplicity and typically not required a previous modification of the support surface [16]. Enzymes can be adsorbed on solid surfaces by different forms of interaction. Enzymes with large hydrophobic areas can be immobilized on

hydrophobic supports through van der Waals forces and entropy changes. The same is true for enzymes with large hydrophilic areas. The sugar residues of glycosylated enzymes can be adsorbed via hydrogen bonds [17].

Enzymes can be immobilized on many materials by physical adsorption. And the Candida Antarctica lipase B should be the most successful commercialization example for physical adsorption immobilization which is Novozym 435 [18,19]. It is the lipase adsorbed on a hydrophobic macroporous polymer which is based on methyl and butyl methacrylic esters and cross-linked with divinylbenzene. Silica-based support for enzyme immobilization are also commonly used materials in such methods, such as silica-based gels [20,21] and diatomaceous earth [22]. Therefore, the adsorption method is generally used in large-scale production, especially in the immobilization of inexpensive enzymes. However, their combination is usually too weak and vulnerable to media influence. So that under the conditions of high reactant and product concentration and high ionic strength, the enzyme is easily desorbed from the support [23].

(2) Ion binding

The ionic bond method uses ionic bonds to bind the enzyme to a support with ion-exchange groups. Moreover, basically, any ion exchanger can act as a support through ion exchange and strong polar interactions [24–26]. The ion exchanger needs to be positively or negatively charged according to the main charge of the enzyme. AS the show in Fig. 1-2 [17].

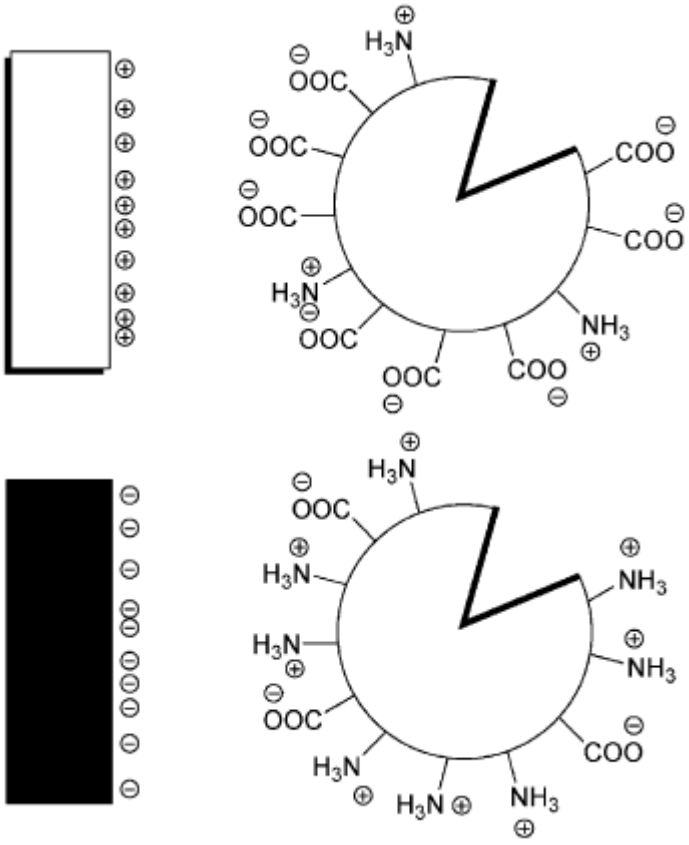

Fig. 1-2. The types of ion exchangers for enzyme immobilization depending on their surface charge [17].

Enzymes can be immobilized on polysaccharides [27], dextran [28], agarose [29], and chitosan [30]. These biopolymers can be modified by different functional groups so that the support has the required charge to realize the ion-binding immobilized enzyme. In addition, metal-chelating supports are also commonly used in ionic bonding method. The metal ions (such as iron [31], copper [32], nickel [33], and cobalt [34], etc.) on the metal-chelating support interact with amino acid residues on the protein surface at multiple points to form a stable complex. The ion binding method is reversible, thus making the support recyclable. However, this combination has a strong dependence on the pH and salt concentration of the medium. Moreover, high salt concentrations can lead to ion exchange and enzyme desorption.

(3) Covalent binding

The covalent binding method refers to the irreversible connection between the

enzyme and the support in the form of a covalent bond. The covalent binding between enzyme and support is tightly fixed which can minimize the enzyme leaching and the enzyme contamination of the product [35]. The formation of multiple covalent bonds between the enzyme and the support will reduce the flexibility and thermal shock of the enzyme, and change its activity. But these covalent bonds prevent protein unfolding and denaturation. Most commonly, the amino and carboxyl groups of enzymes are used for covalent binding method [36,37]. This method generally requires the support to be pretreated to have specific functional groups and then activated by a bifunctional crosslinking agent. Finally, the activated support combines with the amino or carboxyl group on the enzyme to form a chemical bond. As shown in Fig. 1-3. The amino group as a nucleophile can react with epoxides or aldehydes to form irreversible immobilization [17]. Carbodiimide is also often used to link the support and the carboxyl or amino group on the enzyme to form an amide bond [38]. However, in the process of immobilization, the immobilization of all enzyme molecules does not occur in a uniform manner. Nevertheless, the multi-point connection method can still tightly connect the enzyme and the support. Therefore, it is a very important part of choosing a dual-functional crosslinking agent [39].

Glutaraldehyde should be the most widely used as a cross-linking agent because it is facile, efficient, and can improve the stability of enzyme by multipoint or multisubunit immobilization [40,41]. The support with primary amino groups can be activated by glutaraldehyde. Then, the glutaraldehyde-activated support reacts with the primary amino groups of enzymes. As shown in Fig. 1-4. For immobilized cellulase, the glutaraldehyde activated support may be sterically hindered because of its spacer arms. However, it can be considered as hetero-functional support that can provide chemical reaction groups and anion exchange, and gives the highest reactivity with the amino groups of the protein [42].

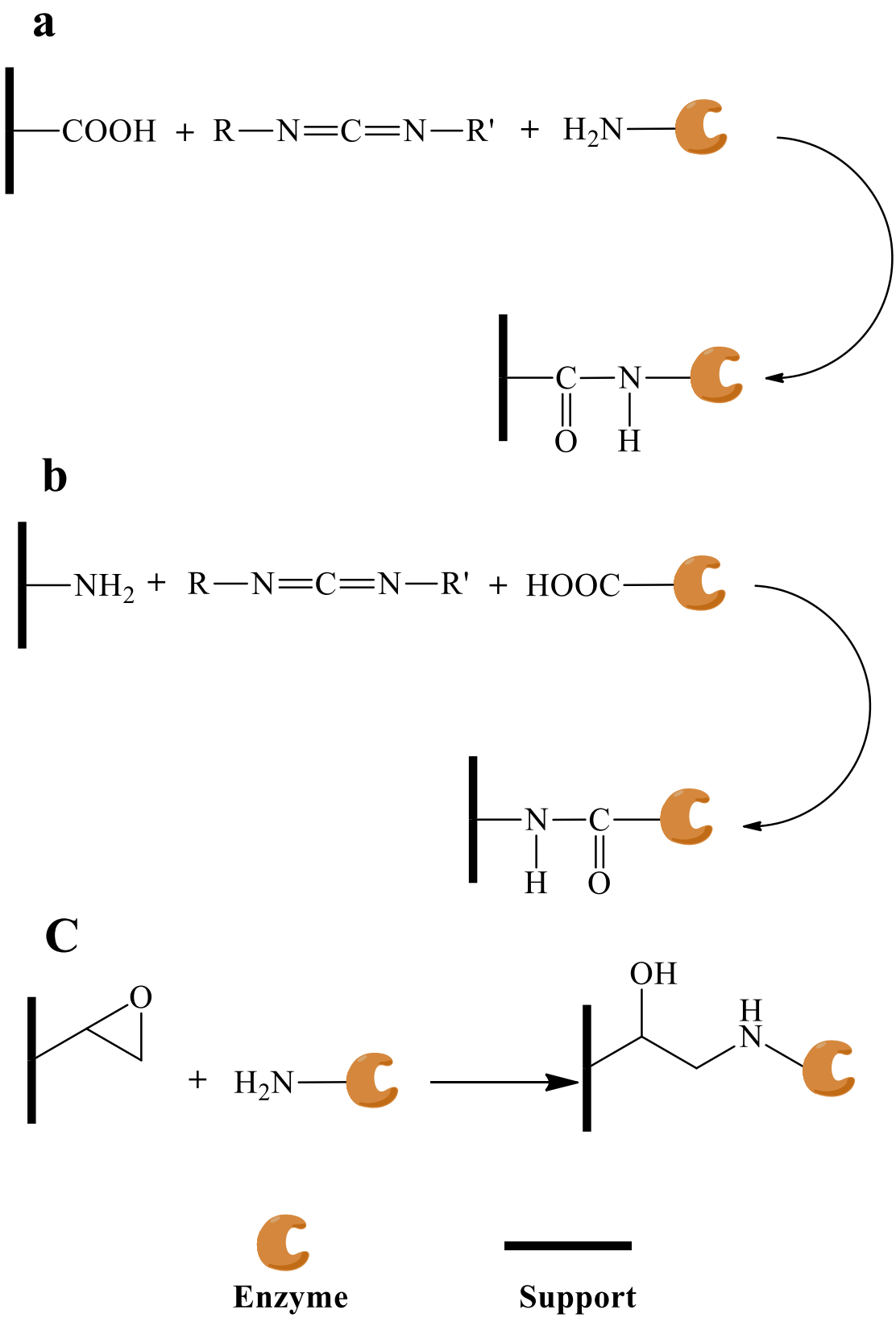

Fig. 1-3 Amino and carboxyl groups on the surface of the enzyme react with the activated support forming covalent bonds.

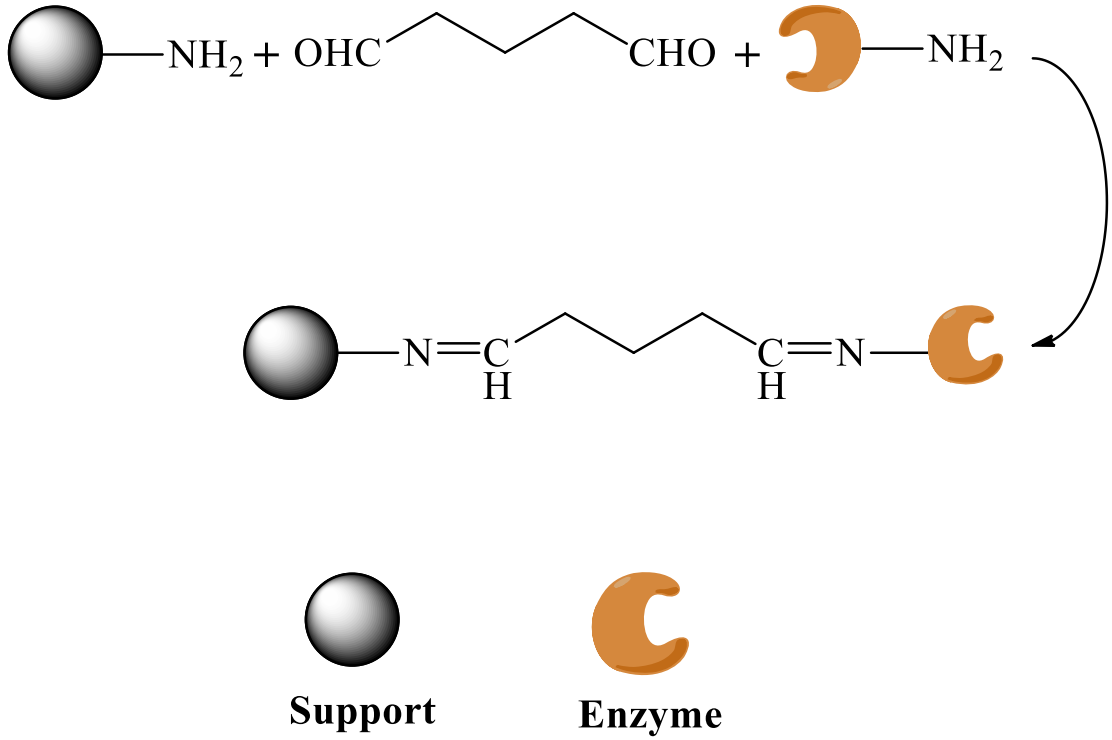

Fig. 1-4 Enzyme immobilization by glutaraldehyde

1.2.2 Entrapment

Entrapment via inclusion of enzymes into a polymer network or a microcapsule. It could be the best method of avoiding any negative influence on the structure of an enzyme after immobilization [43]. Because the conditions for preparing the immobilized enzyme by the embedding method are mild. Inside support, the enzyme itself will not undergo a chemical combination reaction and will not change the structure of the enzyme. Therefore, higher enzyme activity recovery can be obtained, and this method is suitable for the immobilization of various enzymes.

The silica sol-gel matrix formed by hydrolysis polymerization is the most commonly used method for enzyme entrapment. A sol-gel prepared from a mixture of $Si(OCH_3)_4$ and $RSi(OCH_3)_3$ can provide a more hydrophobic matrix for enzyme entrapment. This method involves higher lipases loading, and the immobilized enzyme exhibit an excellent catalysts in the kinetic resolution of chiral alcohols and amines and the reusability [44–46]. Synthetic hydrogels PVA is also the common use for enzyme

entrapment. Veronese et al prepared PVA hydrogels that are stable in aqueous solutions by freezing and thawing. The conjugate of pretreated enzyme and PEG immobilized on PVA hydrogel can effectively reduce lipase leakage. The immobilized enzyme can maintained its activity in organic solvents. And it can reduce the restraint of diffusion by cutting the matrix into micron slices, thereby improving activity [47].

The entrapment method for enzyme immobilization is fast, cheap, and usually involves mild conditions. However, the entrapment immobilized enzyme is only suitable for the catalytic reaction of small molecule substrates and products and is not suitable for the reaction of large molecule products or substrates. Moreover, diffusion resistance can cause changes in the kinetic behavior of the enzyme and reduce its activity [48]. And the enzyme leakage is also one of the drawbacks using a sol-gel technique for the enzyme entrapment [49].

1.2.3 Crosslink

Cross-linking is an extreme case of covalent bonding. It relies on using bifunctional reagents to promote aggregation between enzyme molecules to form a network structure [50,51]. This network structure is a form of the immobilized enzyme because it is insoluble in water. And it is a support-free method for enzyme immobilization. Commonly used bifunctional reagents are glutaraldehyde [52] and maleic anhydride [53]. The free amino group, carboxyl group, imidazole group, and sulfhydryl group of the enzyme can undergo a crosslinking reaction with the cross-linking agent [54]. Enzymes exist in different states before cross-linking, such as dissolution, crystallization, spray drying, and aggregation [55–57]. After cross-linking and immobilization, they respectively formed cross-linked dissolved enzymes (CLEs), cross-linked enzyme crystals (CLECs), cross-linked spray-dried enzymes (CSDEs), and cross-linked enzyme aggregates (CLEAs). AS the show in Fig. 1-5 [58].

CLEAs and CLECs are the support-free immobilized enzyme, which can effectively avoid the dilution of activity caused by the use of solid support. Therefore, in the process of catalysis, its enzyme activity is highly concentrated. In addition, this

technology can also improve the stability of the enzyme. Rehman [59] used Ethylene glycol-bis [succinic acid N-hydroxysuccinimide] (EG-NHS) as cross-linking agents to immobilized lipase. The EG-NHS aggregates shown hydrolytic (52.08 ± 2.52%) and esterification (64.42%) activities recover and a significant improvement in the thermal resistance comparing with free enzyme. In addition, after 10 reusability cycles, The EG-NHS aggregates preserved 70.9% of its original activity. However, there are several limitations in the use of CLEs, such as poor reproducibility, low mechanical stability, and difficulties in handing the gelatinous CLEs [60].

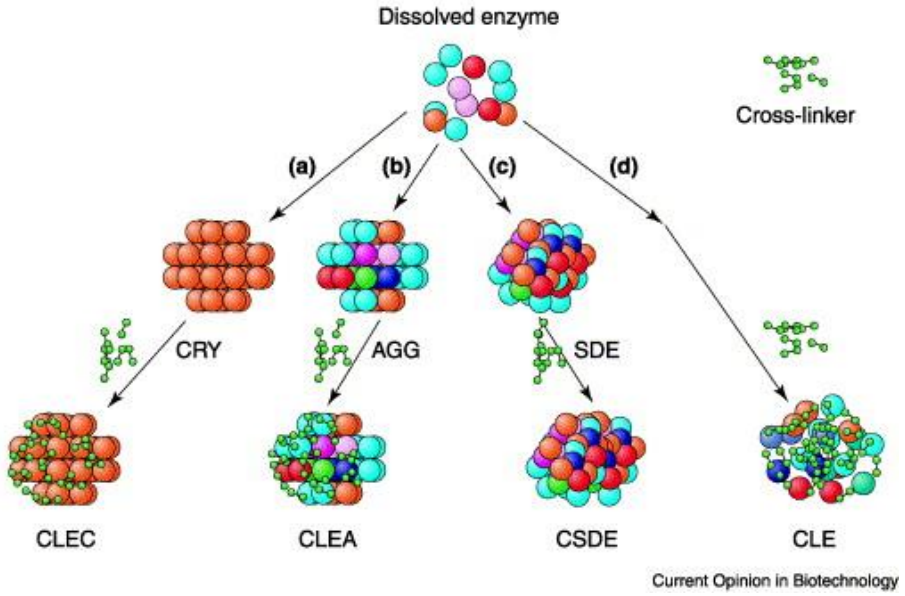

Fig. 1-5 The types of production of support-free immobilized enzymes: (a) crystallization; (b) aggregation; (c) spray-drying; (d) direct cross-linking. AGG, aggregates; CRY, crystals; SDE, spray-dried enzyme [58].

1.3 Porous materials for enzyme immobilization

The nature of the immobilized enzyme is determined by the nature of the enzyme and the support material. The interaction between the enzyme and the support provides specific chemical, biochemical, mechanical, and kinetic properties to the immobilized enzyme [61,62]. Therefore, it is very important to choose a suitable support. Porous materials are commonly using for enzyme immobilization. Because it is thermally, mechanically and chemically stable and insoluble in the solution for the immobilization

and catalytic process. In addition, it also can provide large surface area for enzyme binding [63,64].

1.3.1 Porous support selection

(1) Mechanical properties

In the process of enzyme immobilization and catalysis, shaking and stirring are generally required to increase the fluidity of the medium. This makes it easier for the enzyme or substrate to diffuse into the support. Therefore, the support should have good mechanical properties to prevent its structure from being damaged during these processes [65]. Silica-based materials, carbon materials, porous glass, zeolites and diatomite should be a very good selection for this part [42].

(2) Particle size

In the immobilization and catalytic process, the particle size of support plays an important role. Larger particles may be easier to obtain and recover. But the poor diffusion of the lager support should be a big problem. For example, the diffusion rate of the substrate is relatively low at low temperature and slow stirring. This also means that the amount of substrate diffused into the support is less than the amount consumed, thus showing the low activity of the immobilized enzyme [66]. However, too small particles may be almost impossible to handle in the industrial conditions [67]. In generally, commonly used porous materials, such as silica-based materials, carbon materials, and zeolites, have particle sizes ranging from hundreds of nanometers to hundreds of microns [68], which should be suitable for enzyme immobilization. Wang [69] et al synthesized mesoporous carbon spheres (MCSs) with controllable particle size and pores. The sphere size of MCSs can be controlled in the range from 10 to 500, which also have large surface area (> 600 m^2/g) and large pore volume (> 1 cm^3/g). The MCSs shown a high adsorption capacities (ca. 1100 mg/g) for α-Chymotrypsin in solution, because of controllable pore size, unique shape and good affinity to

biomolecules. Gustafsson [70] et al synthesized three kinds of mesoporous silica with the same pore size (9 nm) and different particle size (1000, 300 and 40 nm) for lipase immobilization. They all exhibited similar enzyme loadings. However, the 300 nm particle was proven to be the most suitable support because it shown a high specific activity.

(3) Pore size and specific surface area

Pore size and specific surface area are the important part of a porous material. Commonly, the larger the pore size, the smaller the specific surface area it is.

For the pore size, it is a direct factor of whether the enzyme can enter the porous material. It should be considered that the pore size must be larger than the enzyme molecule to ensure that the enzyme molecule can enter the inside of the porous material, and it is necessary to prevent the enzyme from blocking the pore structure [71]. As the shown in Fig. 1-6 [42]. Selecting suitable enzymes for immobilization according to the pore size of the porous material can effectively increase the enzyme loading. In addition, during the catalysis process, the diffusion of the substrate should also be considered. If the size of the substrate is larger than or nearly to the pore size of the porous material, the substrate cannot effectively enter the pores through diffusion to participate in the enzymatic reaction [72]. Which could lead to low enzyme activity. As the shown in Fig. 1-7 [42].

For the specific surface area, it is the direct factor of the loading amount of the support (under the enzyme that can pass through the pores of the support smoothly). Large specific surface area can provide more specific areas for enzyme immobilization [17,73]. However, the loading of enzymes should not exceed a specific area, otherwise it is easy to cause enzyme stacking. Usually, a large specific surface area of support is an economical point for enzyme immobilization.

Therefore, it is necessary to consider the pore size and specific surface area of the support for enzyme immobilization.

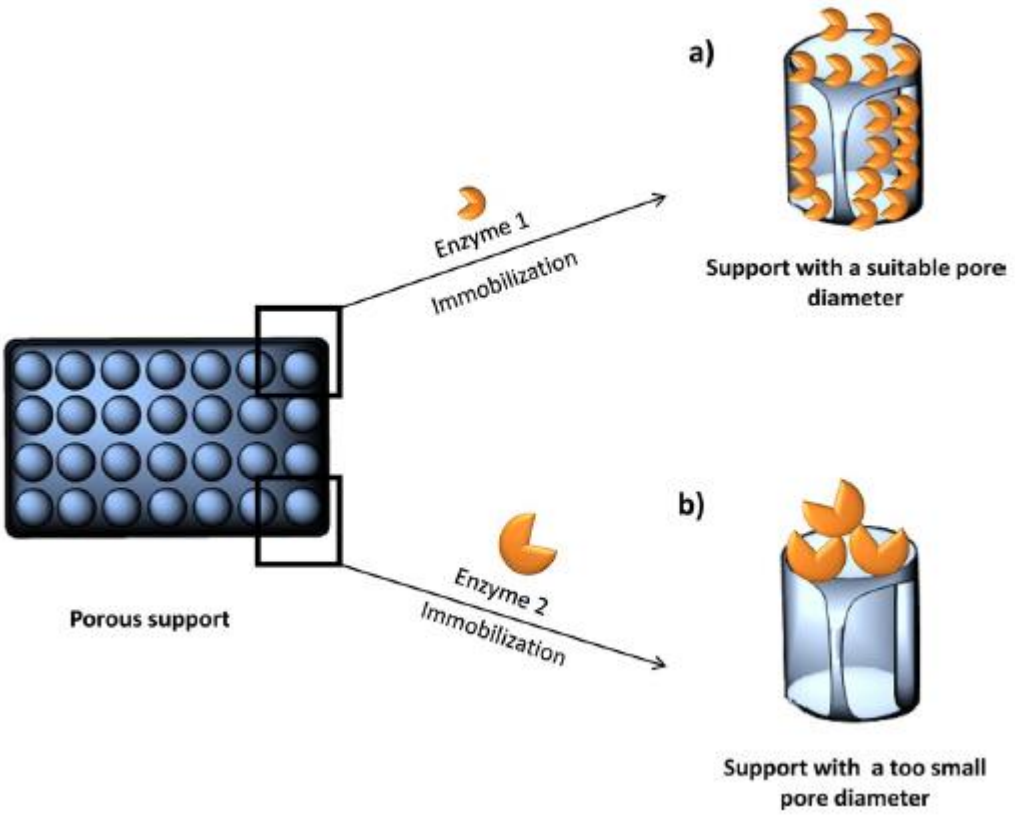

Fig. 1-6 Effect of pore size on the loading capacity of the support for enzyme immobilization [42]

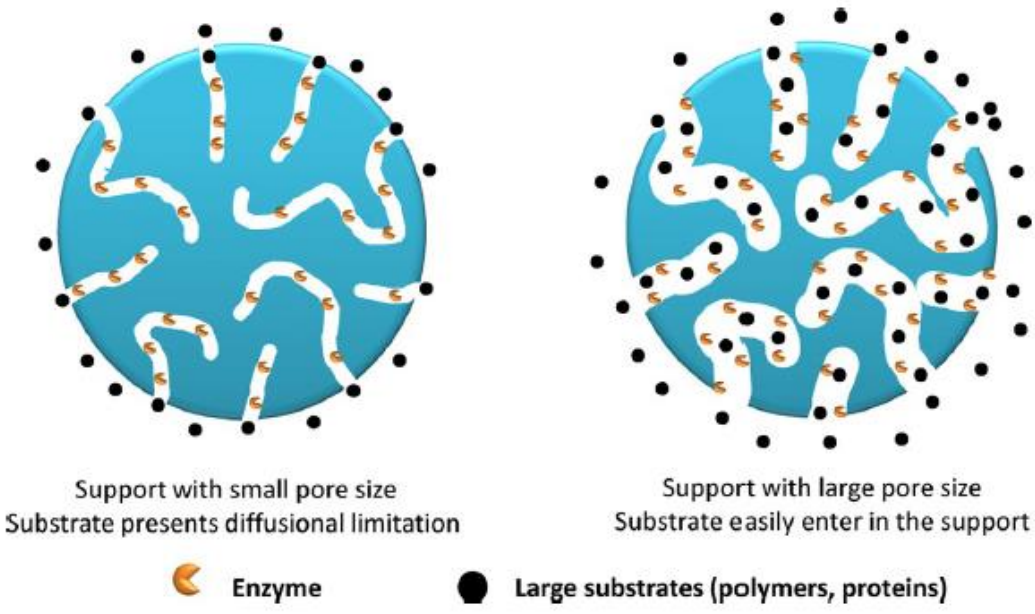

Fig. 1-7 Effect of pore size on the diffusional limitations of substrates [42]

(4) Surface chemistry

The surface chemistry of the support is also an important part because it affects the strength of the interaction between the enzyme and the support surface [17]. The surface properties of the support, such as hydrophilic/hydrophobic surface, positive/negative charge and surface functional groups, determine the method of the enzyme immobilization. Corresponding discussions have been carried out in the "1.2.1 Binding to a support" section. In order to better bind enzymes, porous materials usually require further chemical modification. Jung [74] et al using mesoporous SBA-15 with two kind of organic linkers, 3-glycidoxypropyltrimethoxysilane (GTS) and with 3-aminopropyltrimethoxysilane and glutaraldehyde (GA-ATS), to discuss the influence of functional linker on the surface on the immobilization amount and enzyme activity of chloroperoxidase (CPO) and glucose oxidase (GOx). The results shown that the enzymes immobilized by organic parts can be stored for several weeks without losing their activity. And the covalent linker provides a strong bond to reduce desorption relative to physical adsorption.

1.3.2 Porous biochar

Biochar is a solid product of pyrolysis of biomass including bagasse, straw and forest residue. The biomass is subjected to a high temperature which will leads a changing in its composition. Moreover, under anaerobic conditions, high-temperature carbonization decomposes part of the biomass but retains most of the carbon content [75,76]. In the pyrolysis process, temperature should be the most important condition. The components of biomass are pyrolyzed at different temperatures and the resulting products as shown in Fig. 1-8 [77]. In general, with the temperature increase, the specific surface area and carbon content of biochar increase. However, the raise in treatment temperature leads to a decrease in both H/C and O/C ratios [78]. This will result in the lack of functional groups on the surface of biochar, which is not conducive to the immobilization of enzymes.

Usually, biochar has a rudimentary porous structure and specific surface area, but it is insufficient for practical use. To be a support, the biochar needs to increase its specific surface area and pore fraction or form reactive functional groups. Therefore, chemical or physical activation always has been used by many researchers for the production of porous biochar. Azargohar [79] et al used biochar as a precursor of activated carbon using physical (steam) and chemical (potassium hydroxide) activation processes. Before activation, the BET surface area of biochar is less than 10 m^2/g. After physical and chemical activation, it became 634 and 783 m^2/g, respectively.

The applications of biochar are very diverse, ranging from soil amendment [80], wastewater treatment [81], and electrode material [82]. However, there are few reports of using porous biochar as a support to immobilize cellulase. Porous biochar has a high specific surface area (~1600 m^2/g), which can provide more specific areas for enzyme immobilization. It also has a wide pore size distribution and can be applied to the immobilization of different types of enzymes. Compared with other materials (mesoporous silica [83], zeolite [84], graphene [85], and metal-organic framework [86]), porous biochar has the advantages of abundant sources, simple preparation method, and cheap. Therefore, porous biochar as a support for immobilization of cellulase has good application potential. However, the insufficient of reactive groups and hydrophilic groups, and inconvenient recovery limit its application in the immobilization of hydrophilic enzymes. Therefore, the overall aim of this thesis work is to solve these problems.

1.4 Aims of this research

As the discussions above, the limitations of free cellulase application including the enzyme inactivation during the hydrolysis process and the difficulty to separate and reuse cellulase from the reaction medium. Immobilization can be used for enhancing the stability and easy recovering and reusing of cellulase. Many solid materials are used as supports for immobilized cellulase. However, there are few reports on the use of porous biochar to immobilize cellulase. Therefore, three kind of functional porous

biochar were designed as supports for cellulase immobilization via covalent bonding or adsorption in this thesis work due to the following reasons:

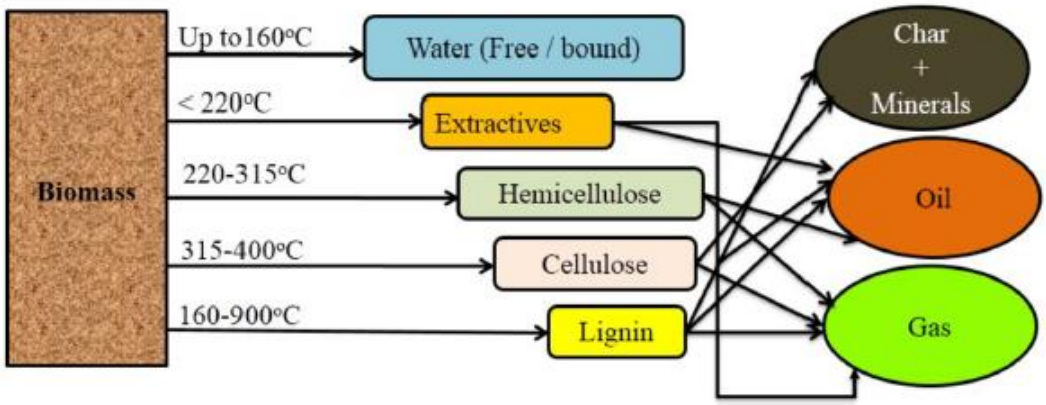

Fig. 1-8 The components of biomass are pyrolyzed at different temperatures and the resulting products [77]

(1) The porous biochar prepared in this work has a high specific surface area (~1600 m^2/g) and polyporous structure (macropores, mesopores and micropores), which could be emerging as a promising support to immobilize enzymes. However, the insufficient of reactive groups and hydrophilic groups limit its application in the immobilization of hydrophilic enzymes. In order to improve the biocompatibility of porous biochar, chitosan is used to modify the supports because it has good hydrophilic, biocompatible, and non-toxicity. In addition, chitosan can provide amino groups to covalently bind with the enzymes. Then the functional chitosan/porous biochar was used as support for cellulase immobilization by GA. The properties of immobilized enzyme was studied.

(2) In order to conveniently and quickly recycle and reuse enzymes, the magnetic material has been paid much attention because it can be easily separated from the reaction system by applying a magnet. Co-precipitation and hydrothermal methods are commonly used to prepare magnetic-based materials. However, it is difficult to use the two methods to prepare the magnetic base biochar because there are few functional groups on the surface of the activated porous biochar. If the iron ions are firstly

dispersed and attached to the biochar, the magnetic particles can be uniformly grown in the biochar after calcination. This should be a good strategy to prepare magnetic porous biochar. Then the magnetic porous biochar was used as support for cellulase immobilization by adsorption. The preparation conditions of the support, the adsorption of cellulase by the support, and the properties of the immobilized enzyme were studied.

(3) Combining the advantages of the first two solutions, the chitosan/magnetic porous biochar was designed to make the support have good biocompatibility and convenient recycling and reuse. And then it was used as a support for the cellulase immobilization via glutaraldehyde. The structure and morphology of the support have been characterized, and the enzymatic properties of free enzyme and immobilized enzyme were evaluated in hydrolyzed carboxyl methyl cellulose sodium, including optimum pH and temperature, kinetic parameters, and reusability.

Reference

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Chapter 2 Materials, experimental methods and characterizations

2.1 Materials

2.1.1 Cellulase

Cellulase, a composite enzyme, mainly compose by endo-l, 4-β-D-glucanase, exo-l, 4-β-D-glucanase and β-glucosidase. Its classification is based on attacking the depolymerization stage of the substrate. Endoglucanases randomly hydrolyze the glycosidic bonds in the amorphous regions of cellulose to produce oligomers with several degrees of polymerization. Then, exoglucanase hydrolyzes the β-1, 4-glycosidic bond of the oligomer to produce cellobiose. Finally, cellobiose is degraded to glucose by β-glucosidase[1]. The cellulase was bought from Meiji Seika Pharma Co., Ltd (Tokyo, Japan).

2.1.2 Sugarcane bagasse

Sugarcane bagasse, an agricultural waste, is used to make porous biochar. Because of its rich source, low price, and hollow structure. After the bagasse is activated by alkali and pyrolysis, biochar with a high specific surface area and the porous structure can be obtained. Sugarcane bagasse was purchased from Guangxi, China.

2.1.3 Chitosan (CS)

Chitosan is obtained from chitin by deacetylation, and it is widely found in nature. It has many advantages, such as non-toxicity, good biocompatibility, and abundant functional groups (amino, hydroxyl and methyl) [2]. The pK_a value of chitosan is about 6.5, which means that the soluble/insoluble transition of chitosan can be easily adjusted by changing the pH value of solution [3]. Thus, chitosan is considered to be an ideal

material for the modification of supports to endow them with abundant functional groups by an easy and low-cost method. Chitosan was purchased from Nacalai Tesque, Inc. (Tokyo, Japan).

Fig. 2-1 image of sugarcane bagasse

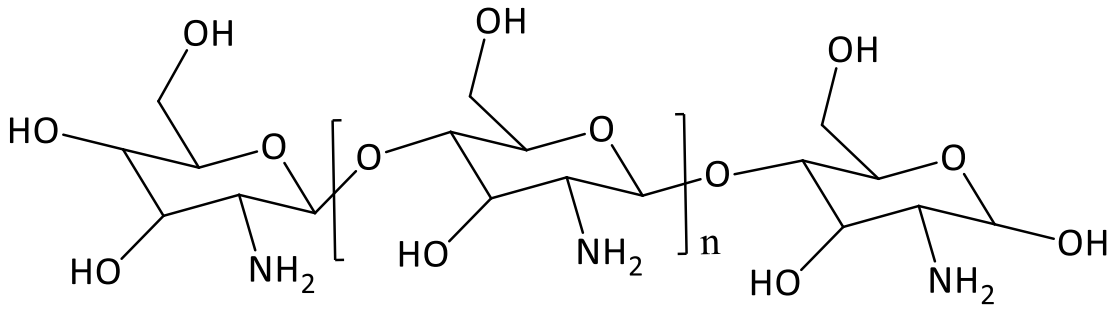


Fig. 2-2 structure of chitosan

2.1.4 Glutaraldehyde (GA)

Glutaraldehyde should be the most widely used as a covalent bonding agent because it is facile, efficient, and can improve the stability of enzyme by multipoint or multisubunit immobilization. The support with primary amino groups can be activated by glutaraldehyde. Then, the glutaraldehyde-activated support reacts with the primary

amino groups of enzymes [4]. Glutaraldehyde was purchased from Nacalai Tesque, Inc. (Tokyo, Japan).

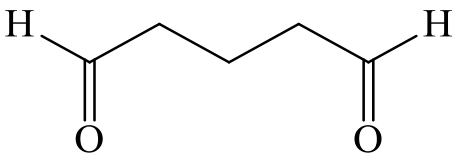


Fig. 2-3 structure of glutaraldehyde

2.1.5 Carboxyl methyl cellulose sodium (CMC)

In this thesis work, we used carboxymethyl cellulose (CMC) as substrate since (CMC) assay has been widely used in the characteristic of the free and immobilized cellulase. The CMC was purchased from Nacalai Tesque, Inc. (Tokyo, Japan).

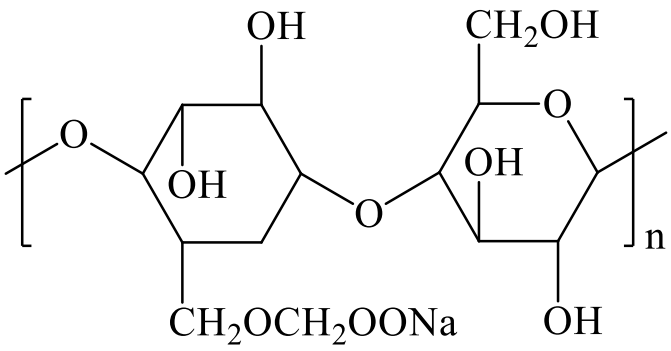


Fig. 2-4 structure of CMC

2.1.6 Bradford reagent

Bradford reagent was used to determine the concentration of cellulase by the Bradford protein assay method. Bradford reagent was purchased from sigma-aldrich co. llc.

2.1.7 Dinitrosalicylic (DNS) reagent

DNS reagent was used to determine the concentration of reducing sugar, which is used to calculate cellulase activity. The preparation method of DNS reagent is as follows:

182 g of potassium sodium tartrate, 21g of NaOH, 6.3 g of 3,5-dinitrosalicylic acid,

5 g of phenol, and 5 g of anhydrous sodium sulfite were dissolved in 1000 mL of distilled water and then placed at room temperature for a week before use.

2.1.8 Other materials

Potassium hydroxide (KOH), hydrochloric acid (HCl, 35-37 wt%), acetic acid (HAc), sodium acetate (NaAc), ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), and ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$) were purchased from Nacalai Tesque, Inc. (Tokyo, Japan).

2.2 Experimental methods

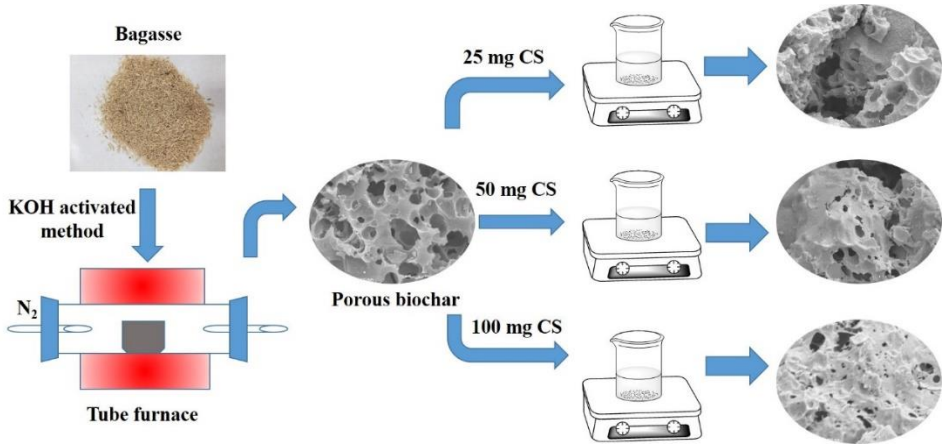
2.2.1 Preparation of porous biochar

Porous biochar was prepared by KOH activation method [5]. Bagasse was ground to pass a 1 mm sieve and immersed in distilled water at 95 °C for 8 h. Then the obtained bagasse, KOH and ethanol with a feeding ratio of 1 g: 1 g: 12 mL were added to a beaker and mixed at 60 °C for 5 h. After drying at 60 °C for 12 h, the treated bagasse was putted into tube furnace for calcination at 800 °C for 2 h (nitrogen protection; heating rate: 10 °C/min) and naturally cooled to room temperature. After grinding (without screening), the product was washed with 12.5 mL of 1.5 M HCl solution for removing alkali and ash. Finally, the obtained porous biochar was filtered and washed with distilled water for several times until pH became neutral and then dried at 60 °C for 24 h. The porous biochar was denoted as C.

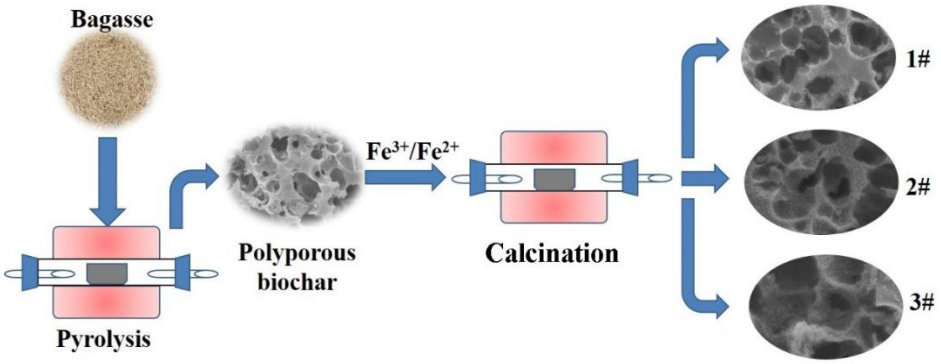
2.2.2 Preparation of functional supports

For the C@CS, different amount of CS was added into 25 mL of 1% (v/v) acetic acid solution with magnetic stirring at room temperature. After the CS dissolved, 0.5 g C was added and mixed rapidly for 0.5 h. After that, 25 mL of 1 M NaOH solution was added. After 30 min, the products were centrifuged and washed with distilled water several times.

(a) C@CS



(b) C/γ-Fe_2O_3



(c) C/γ-Fe_2O_3@CS

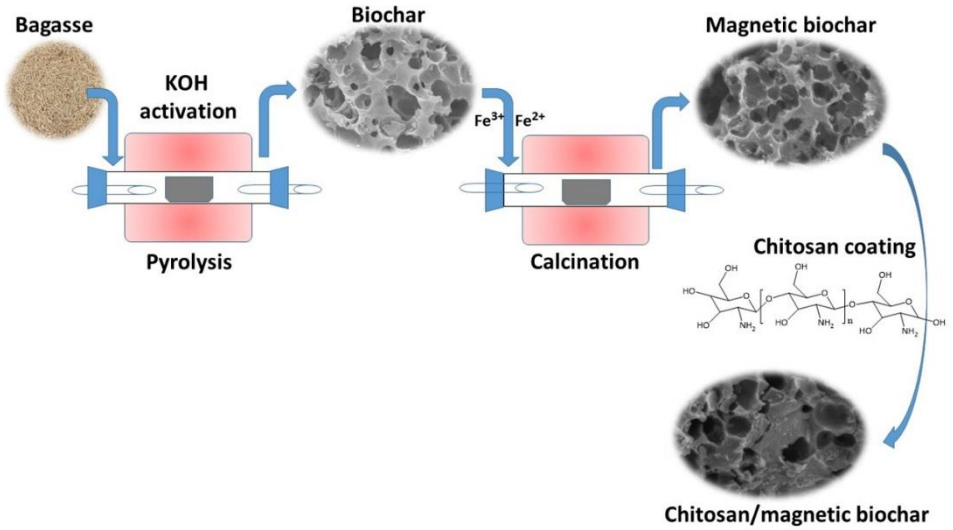


Fig. 2-5 Schematic diagram of preparation process of C@CS (a), C/γ-Fe_2O_3 (b) and C/γ-Fe_2O_3@CS (c).

For the C/γ-Fe_2O_3, 0.1 g C was added in 2 mL ethanol solution (containing $FeCl_3 \cdot 6H_2O$ and $FeCl_2 \cdot 4H_2O$). The mixture was dried at 70 °C for 10 min in tube furnace, and then the temperature was raised to 400~700 °C for 10~120 min (N_2 protection, heating rate of 10 °C/min).

For the C/γ-Fe_2O_3@CS, the optimal value in chapter 4 was selected and further modified with chitosan. 0.5 g C/γ-Fe_2O_3 was added into 25 mL of 1% (v/v) acetic acid solution (containing 50 mg chitosan) with a strong stirring at room temperature for 30 min, and then, it was mixed with 25 mL of 1M NaOH solution. The products were recovered by a magnetic and washed with distilled water for 5 times. And it was denoted as C/γ-Fe_2O_3@CS.

2.2.3 Immobilization of cellulase on functional supports

(1) C@CS immobilized cellulase via GA

The C@CS was dispersed in 25 mL of 2.5% (v/v) GA solution with a magnetic stirring at room temperature for 2.5 h, respectively. The products were washed with distilled water for 3 times and 0.1 M HAc-NaAc buffer solution (pH value = 5) for once. Then, the samples were dispersed in 25 mL of 4 mg/mL cellulase solution (pH value = 5) with a slow magnetic stirring at room temperature for 12 h. Finally, the products were centrifuged and washed with 25 mL of 0.1 M HAc-NaAc buffer solution (pH value = 5) for 2 times and the supernatants were retained to determine the residual cellulase concentration. The immobilized enzymes were stored at 4 °C.

(2) C/γ-Fe_2O_3 immobilized cellulase via adsorption

A certain amount of support was added in 10 mL pH value= 5 cellulase solution. The solution was placed into an air bath shaker and shaken with an oscillation speed of 120 r/min for 12 h. During this process, 0.2 mL mixture was taken out. After separation by magnet, 0.05 mL supernatant was used for the determination of residual cellulase concentration at 30 min, 1 h, 3 h, 6 h, 9 h and 12 h, respectively.

(3) C/γ-Fe_2O_3@CS immobilized cellulase via GA

0.5 g of C/γ-Fe_2O_3@CS was dispersed in 25 mL of 2.5% (v/v) GA solution with a magnetic stirring at room temperature for 2.5 h, respectively. The products were washed with distilled water for 3 times and 0.1 M HAc-NaAc buffer solution (pH = 5) for once. The activated support was put in 25 mL of 4 mg mL^{-1} cellulase solution (400 mg of cellulase powder was dissolved in 100 mL of 0.1 M pH=5 HAc-NaAc buffer solution at room temperature) with a low stirring at room temperature for 12 h. The products were washed with 0.1 M pH=5 of HAc-NaAc buffer solution for three times and recovered by a magnetic. The immobilized cellulase was stored at 4 ºC.

2.2.4 Determination loading amount of cellulase

The amount of cellulase in the supernatant before and after immobilization were determined by the Bradford method [6].

$$\text{Immobilized amount} = C_0V_0 - C_1V_1$$

where C_0 and V_0 is the concentration and volume of initial cellulase solution, respectively. The C_1 and V_1 is the concentration and volume of cellulase solution after immobilization, respectively.

2.2.5 Activity assays

The cellulase activity was evaluated according to updated IUPAC method [7]. Specifically, the immobilized cellulase containing 0.2 g cellulase was mixed with 10 mL 1% CMC buffer solution before being preheated at hydrolysis for 5 min. During this process, the pH value and temperature of cellulase were optimum. For pH value, the activity was analyzed at 50 °C (recommended temperature by producer) with the pH values from 3 to 7. For temperature, the activity was analyzed at 30 to 70 °C at pH value = 4 (the optimum pH value). After being hydrolyzed for 30 min and inactivated for 5 min in boiling water, the immobilized cellulase and products were separated by centrifugal separation. Then, 1 mL supernatant was mixed with 2 mL DNS solution and

the resulting solution was heated by boiling water for 5 min. Finally, 10 mL distilled water was added to dilute the products. The amount of glucose was determined by a UV-vis spectrophotometer at 540 nm. The International Unit of cellulase activity (IU/mg cellulase) is defined as a certain amount of cellulase that hydrolyzes CMC to produce 1 μmol glucose per minute.

2.2.6 Reusability assays

For the practical applications, enzymatic hydrolysis for a longer period of time should be considered [8]. Immobilized cellulase was dissolved in 1% CMC solution (cellulase : CMC = 3 : 100) and the hydrolysis was proceeded at optimum pH value and temperature for 24 h. After separation, the immobilized enzyme was added into a fresh substrate solution, and this process was repeated 10 times.

2.3 Characterizations

2.3.1 Scanning electron microscopy (SEM)

The samples covered with an ultra-thin layer of a gold/palladium alloy were analyzed by scanning electron microscope (SEM, Hitachi S 4300, Japan).

2.3.2 Brunauer-Emmett-Teller (BET)

The specific surface areas of the composites were determined by Brunauer-Emmett-Teller (BET) method, nitrogen adsorption at 77 K using ASAP 2020 analyzer (Micromeritics, USA).

2.3.3 Fourier transform infrared spectroscopy (FTIR)

The chemical structures of the nanoparticles were confirmed by Fourier transform infrared (FTIR, IN10MX, Thermo Fischer Scientific, USA) spectroscopy using KBr pellets. The FTIR spectra were recorded in the range of 4000 to 400 cm^{-1}.

2.3.4 X-ray diffraction (XRD)

The X-ray diffraction (XRD) patterns were recorded in the range of 2θ = 5 to 90° by step scanning with a PANalytical X'Pert Pro X-ray diffractometer with a Cu Kα radiation source (λ=0.154 nm).

2.3.5 Vibrating sample (VSM)

The magnetism of magnetic supports were determined by vibrating-sample magnetometer (VSM, Riken Denshi Co. Ltd., Japan).

2.3.6 UV visible spectroscopy

The cellulase and glucose concentration were determined by UV spectrophotometer (UV-vis, U-5100, Japan).

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Chapter 3 Porous biochar/chitosan composites for high-performance cellulase immobilization by glutaraldehyde

3.1 Introduction

As a kind of complex enzyme, cellulase consists of endoglucanase, exoglucanase and β-glucosidase, which is plays an important role in the biodegradation of cellulose to glucose [1]. This is because the enzymatic hydrolysis could be effectively and specifically conducted under mild conditions. However, there are several drawbacks that limit its application. For example, cellulase shows a low stability under the changed environmental conditions such as pH value, temperature, and ionic strength, and it is difficult to be reused [2]. Thus, the enzymes are required to be immobilized for easily recycling in practical application [3–5]. The immobilization of enzyme on a solid supports could improve the stability of enzyme and easily recover [6–9].

The immobilization methods can be divided into physical adsorption and covalent bonds. Physical adsorption includes van der Waals force, ionic bonds, and the hydrogen bonds between solid materials and enzymes [2]. It is convenient and rarely changes the structure of enzyme, thus retaining mostly activity of the enzyme. However, during the hydrolysis process, the stability of the immobilized enzyme would be reduced, and the enzyme is easily desorbed from the support because of the weak interaction force between the enzyme and the support. Covalent bonds between solid materials and enzyme could provide stronger interaction, which endows the enzyme more stable and reusable [3]. This gives a more effective way in practical application.

Covalent bond is always formed by a chemical reaction between the activated support and the surface functional groups (-COOH, -NH_2, -SH) of the enzyme [10]. Glutaraldehyde is a commonly used bi-functional reagent in the covalent enzyme immobilization method. The glutaraldehyde-activated supports mainly react with the

primary amino groups of enzyme under a mid-condition [11]. However, each glutaraldehyde molecule could contain one or two amino groups, which indicates that there should be an ionic exchanger on the support [12]. Therefore, the glutaraldehyde-activated support could ionically adsorb enzyme by the primary amino groups before the covalent reaction.

In order to provide high activity, the support having a large specific surface area is necessary. In recent years, popular materials such as graphene [13], mesoporous silica [14], metal organic framework materials [15], and zeolites [16] are well applied in the immobilization of enzymes. However, such materials are complicated to be prepared, which normally requires precision and are highly cost. Especially, most mesoporous materials are used to immobilize enzyme by physical adsorption. The immobilization process is highly impeded by the pore size of the support. Therefore, a multi-functional support with a wide range of sources and easy access could get a lot of interest. Recently, porous biochar attracts much attention in the field of waste water treatment and carbon-based energy storage material [17–20]. The biochar is easily prepared from many kinds of renewable materials including leaves, straw, and bamboo by a carbonization process. Moreover, porous biochar has a high specific surface area (~1600 m^2/g), porous structure (polyporous structure), and surface oxygen-containing functional groups [21–25], which is an ideal support for enzyme immobilization. For the glutaraldehyde activation, a further amino modification is needed for the porous biochar. Chitosan is obtained from chitin by deacetylation, and it is widely found in nature. It has many advantages, such as non-toxicity, good biocompatibility, and abundant functional groups (amino, hydroxyl and methyl) [26]. The pKa value of chitosan is about 6.5, which means that the soluble/insoluble transition of chitosan can be easily adjusted by changing the pH value of solution [27]. Thus, chitosan is considered to be an ideal material for the modification of supports to endow them with abundant functional groups by an easy and low-cost method. By now, many chitosan-based supports have been designed for enzyme immobilization, including natural materials [28,29] and synthetic materials [2,30]. However, porous biochar/chitosan support for enzyme

immobilization has been rarely reported. On the one hand, porous biochar with high specific surface area and rich pore structure can provide more active sites for enzyme immobilization. On the other hand, porous biochar has a wide range of sources, which means it is easy to be obtained and economical. With the assistance of chitosan, porous biochar/chitosan support for enzyme immobilization should be expected.

In this work, porous biochar was prepared from sugarcane bagasse via carbonization and KOH activation method. The prepared biochar was further coated with different amounts of chitosan by changing the pH values of the solution. Finally, the porous biochar/chitosan complex (C@CS) was applied to immobilize cellulase by covalent bonds using glutaraldehyde (GA) as a linker. The C@CS containing different mass of chitosan presented different morphology. The effects of chemical characteristic, morphology and practical size of the support on the cellulase loading amount, activity recover and recycle were discussed.

3.2 Experimental

3.2.1 Materials

Sugarcane bagasse was purchased from Guangxi, China. Potassium hydroxide (KOH), hydrochloric acid (HCl, 35-37 wt%), chitosan (CS), acetic acid (HAc), sodium acetate (NaAc), glutaraldehyde (GA) and carboxyl methyl cellulose sodium (CMC) were purchased from Nacalai Tesque, Inc. (Tokyo, Japan). Cellulase was bought from Meiji Seika Pharma Co., Ltd (Tokyo, Japan).

3.2.2 Preparation of porous biochar

Porous biochar was prepared by KOH activation method [31]. Bagasse was ground to pass a 1 mm sieve and immersed in distilled water at 95 °C for 8 h. Then the obtained bagasse, KOH and ethanol with a feeding ratio of 1 g: 1g: 12 mL were added to a beaker and mixed at 60 °C for 5 h. After drying at 60°C for 12h, the treated bagasse was putted into tube furnace for calcination at 800 °C for 2 h (nitrogen protection; heating rate:

10 °C/min) and naturally cooled to room temperature. After grinding (without screening), the product was washed with 12.5 mL of 1.5 M HCl solution for removing alkali and ash. Finally, the obtained porous biochar was filtered and washed with distilled water for several times until pH became neutral and then dried at 60 °C for 24 h. The porous biochar was denoted as C.

3.2.3 Preparation of C@CS

Different amount of CS (25, 50, 100 mg) was added into 25 mL of 1% (v/v) acetic acid solution with a magnetic stirring at room temperature. After the CS dissolved, 0.5 g C was added and mixed rapidly for 0.5 h. After that, 25 mL of 1 M NaOH solution was added. After 30 min, the products were centrifuged and washed with distilled water for several times. The corresponding product was denoted as C@CS25, C@CS50 and C@CS100, respectively.

3.2.4 Preparation of C@CS immobilized cellulase

The C@CS25, C@CS50, and C@CS100 were dispersed in 25 mL of 2.5% (v/v) GA solution with a magnetic stirring at room temperature for 2.5 h, respectively. Then, the products were washed with distilled water for 3 times and 0.1 M HAc-NaAc buffer solution (pH value = 5) for once. The resulting product was denoted as C@CS25-CHO, C@CS50-CHO, and C@CS100-CHO, respectively.

For cellulase immobilization, the above C@CS-CHO was dispersed in 25 mL of 4 mg/mL cellulase solution (pH value = 5) with a slow magnetic stirring at room temperature for 12 h. Then, the products were centrifuged and washed with 25 mL of 0.1 M HAc-NaAc buffer solution (pH value = 5) for 2 times and the supernatants were retained to determine the residual cellulase concentration. The immobilized enzymes were stored at 4 °C. The cellulase loading amount was determined by the Bradford protein assay method and calculated from the following equation:

$$\text{Immobilized amount} = C_0V_0 - C_1V_1$$

$$\text{Immobilization yield} = ((C_0V_0\text{-}C_1V_1)/C_0V_0)\times 100\%$$

Where C_0 and V_0 is the concentration and volume of initial cellulase solution, respectively. The C_1 and V_1 is the concentration and volume of cellulase solution after immobilization, respectively.

3.2.5 Characterizations

The morphologies of supports were analyzed by scanning electron microscope (SEM, Hitachi SU-70, Japan), and the particle sizes were analyzed by particle size analyzer (MT3000II SERIES, Nikkiso, Japan). The specific surface areas of the composites were determined by Brunauer-Emmett-Teller (BET) method, nitrogen adsorption at 77 K using ASAP 2020 analyzer (Micromeritics, USA). The chemical structures of the samples were confirmed by Fourier transform infrared spectroscopy (FT-IR, iN10MX, Thermo Fischer Scientific, USA). The cellulase and glucose concentration were determined by UV spectrophotometer (UV-vis, U-5100, Japan), and the chemical compositions were investigated by X-ray photoelectron spectroscopy (XPS, ULVAC-PHI) using Al Kα radiation source.

3.2.6 Activity assays

The cellulase activity was evaluated according to updated IUPAC method [32]. Specifically, the immobilized cellulase containing 0.2 g cellulase was mixed with 10 mL 1% CMC buffer solution before being preheated at hydrolysis for 5 min. During this process, the pH value and temperature of cellulase were optimum. For pH value, the activity was analyzed at 50 °C (recommended temperature by producer) with the pH values from 3 to 7. For temperature, the activity was analyzed at 30 to 70 °C at pH value = 4 (the optimum pH value). After being hydrolyzed for 30 min and inactivated for 5 min in boiling water, the immobilized cellulase and products were separated by centrifugal separation. Then, 1 mL supernatant was mixed with 2 mL DNS solution and the resulting solution was heated by boiling water for 5 min. Finally, 10 mL distilled water was added to dilute the products. The amount of glucose was determined by a UV-vis spectrophotometer at 540 nm. The International Unit of cellulase activity

(IU/mg cellulase) is defined as a certain amount of cellulase that hydrolyzes CMC to produce 1 μmol glucose per minute. All experiments were carried out for three times and took the average to plot graphs with standard deviation errors.

3.2.7 Reusability assays

For the practical applications, enzymatic hydrolysis for a longer period of time should be considered [2]. Immobilized cellulase was dissolved in 1% CMC solution (cellulase : CMC = 3 : 100) and the hydrolysis was proceeded at optimum pH value and temperature for 24 h. After separation, the immobilized enzyme was added into a fresh substrate solution, and this process was repeated 10 times.

3.3 Results and discussion

3.3.1 Preparation and characterization of supports

The supports were prepared by a sample method as showed in Fig. 3-1. Biomass is commonly used as a precursor for preparation of porous biochar because it is easily obtained from agriculture and industry. Also, there are two important factors for using lignocellulosic materials to prepare porous biochar, i.e., low inorganic content and easy to be activated [33]. Sugarcane bagasse, an agricultural by-product, is rich in cellulose and has a porous hollow structure; therefore it is an ideal material to prepare porous biochar by a sample alkali activation and pyrolysis [34]. The C@CS was obtained after changing the pH values of solution. Because the pKa value of chitosan was 6.5, chitosan would separate out and adhere to the surface of porous biochar by adding NaOH solution. Finally, the C@CS was grafted with aldehyde group by GA, and cellulase was immobilized by a covalently bound (Schiff base) between support and cellulase.

In general, the methods of enzyme immobilization onto the porous biochar contain adsorption and covalent bonding. Porous biochar can selectively adsorb different types of enzymes because of the hydrophilic/hydrophobic and acidic/basic properties. At the same time, the hydroxyl, carboxyl and π electrons provided by the aromatic ring in

biochar have an important effect on the adsorption performance [35–38]. However, for covalent bonding, the porous biochar requires further surface modification because the available functional groups are little. After coated with chitosan-base, the enzyme can be immobilized both by adsorption and covalent bonding via activation by a cross-linking agent [39]. On the other hand, for a water-soluble enzyme, chitosan-base supports can provide a good biocompatibility and a large number of functional groups, which would contribute to the activity and stability of the immobilized enzyme [2,28].

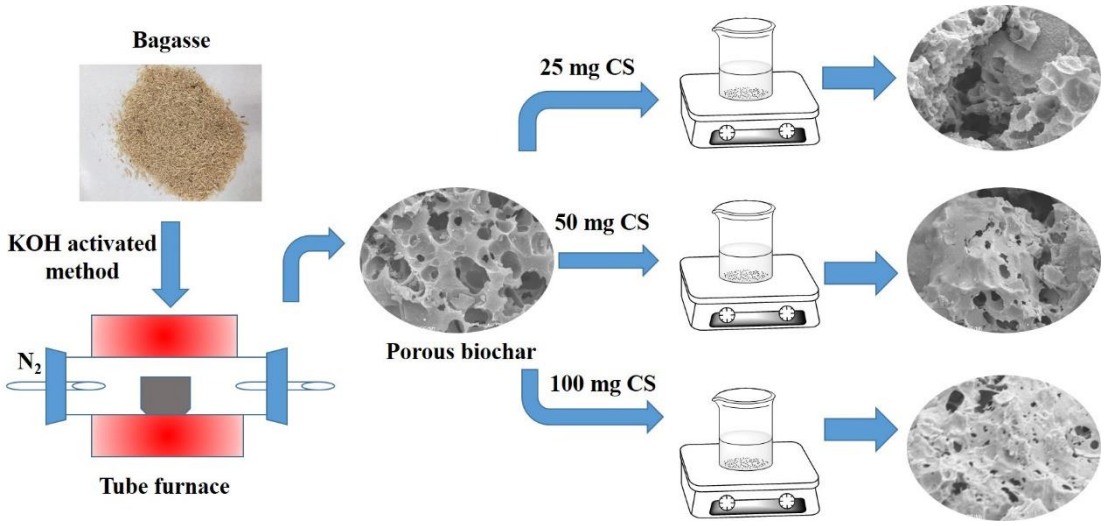

Fig. 3-1 Schematic diagram of preparation process of C@CS supports.

The morphology and particle size of support would affect the enzyme loading amount, activity recovery and reusability [15,40]. The morphology and particle size distribution of C, C@CS25, C@CS50 and C@CS100 are given in Fig. 3-2. For porous biochar (Fig. 3-2a, e), a clearly porous structure was found in the biochar; an allium-giganteum-like microstructure was shown, which is similar to the reported literature [41]. The particle size of porous biochar (Fig. 3-2e) was ranged from 1 to 200 μm and its D50 was 29.17 μm. Compared with that of porous biochar, the SEM images of C@CS supports showed that there was a layer covering on the surface of the biochar (Fig. 3-2b-d; f-h), indicating that the porous biochar was coated with chitosan. The particle size distribution of C@CS25, C@CS50 and C@CS100 were given in Fig. 3-2j-l, and their D50 were 35.78, 41.91 and 53.29 μm, respectively. The particle size

slightly increased as the chitosan amount increased, which might be resulted from two reasons: (1) the thickness of chitosan layer coated on the porous biochar became thicker; (2) a part of small porous biochar were reunited while being coated with chitosan. Moreover, the allium-giganteum-like microstructure was gradually covered as the increased amount of chitosan. In addition, for C@CS25, the porous structures were adequately covered by chitosan. For C@CS50 and C@CS100, most of chitosan were covered above the holes, and formed a chitosan layer. However, there are lots of pores on the chitosan layer.

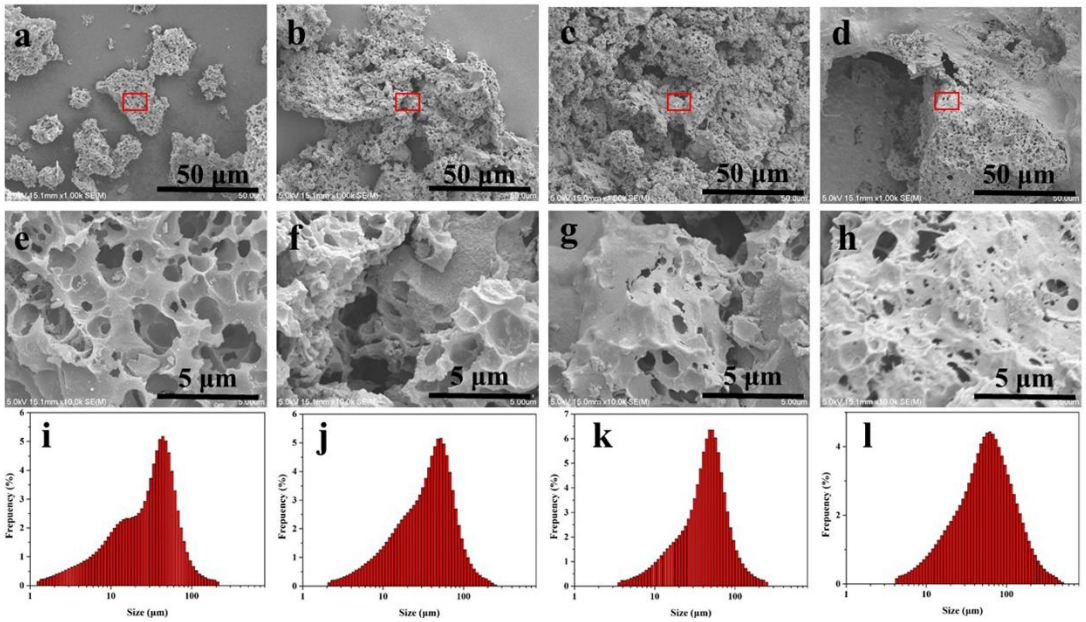

Fig. 3-2 The SEM images of C (a, e), C@CS25 (b, f), C@CS50 (c, g) and C@CS100 (d, h); and the particle size distribution of C (i), C@CS25 (j), C@CS50 (k) and C@CS100 (l).

Fig. 3-3 shows N_2 adsorption-desorption isotherm and pore size distribution by BJH method of the samples. The N_2 adsorption-desorption isotherm of biochar exhibits a combined I/IV sorption isotherm, which illustrates that the biochar has a polyporous structure [34]. As given in Table 3-1, the biochar shows excellent specific surface area (1620.06 m^2/g). However, after the modification with chitosan, the S_{BET} and Vt have different degrees of decline. This is because the coated chitosan on the surface of biochar would block some pore structures of the biochar. Regarding C@CS25,

C@CS50 and C@CS100, as the modifier increased, the SBET of composite increased. It is likely due to the formation of chitosan layer. As proved from the SEM images, under a certain amount of chitosan, a new pore structure was formed while the modifying process of porous biochar. The pore structures increased with the increase of adding amount of chitosan.

The functional groups of C (a), C@CS25 (b), C@CS25-CHO (c), C@CS25-cellulase (d) and cellulase (e) were determined by FT-IR. Because the functional groups of C@CS50 and C@CS100 are similar to C@CS25, they were not discussed in this part. As shown in Fig. 3-4, the C=C resonance vibration and the C-O stretching vibration [42] of C were at 1570 and 1100 cm^{-1}. For C@CS25, the methylene stretching vibrations at 2924 and 2856 cm^{-1} were attributed to the chitosan layer. After treatment with glutaraldehyde, a new vibration peak appeared at 1638 cm^{-1}, which should be the Schiff base. In addition, two characteristic amide stretching vibrations of cellulase at 1648 and 1560 cm^{-1} were found in these immobilized enzyme, which suggests that the cellulase was successfully immobilized onto the support [29].

The chemical compositions of porous biochar, C@CS25, C@CS50 and C@CS100 determined by XPS are given in Table 3-2. The main components of porous biochar are C and O. For C@CS, the N content increased when the chitosan amount increased, showing more chitosan was attached on the surface of the porous biochar.

Table 3-1. BET surface area (SBET), average pore size (Pa) and total pore volume (Vt)

Samples	SBET (m^2g^{-1})	Pa (nm)	Vt (m^3/g)
C	1620.06	2.55	0.478
C@CS25	674.54	4.12	0.111
C@CS50	838.81	3.69	0.130
C@CS100	871.60	3.10	0.102

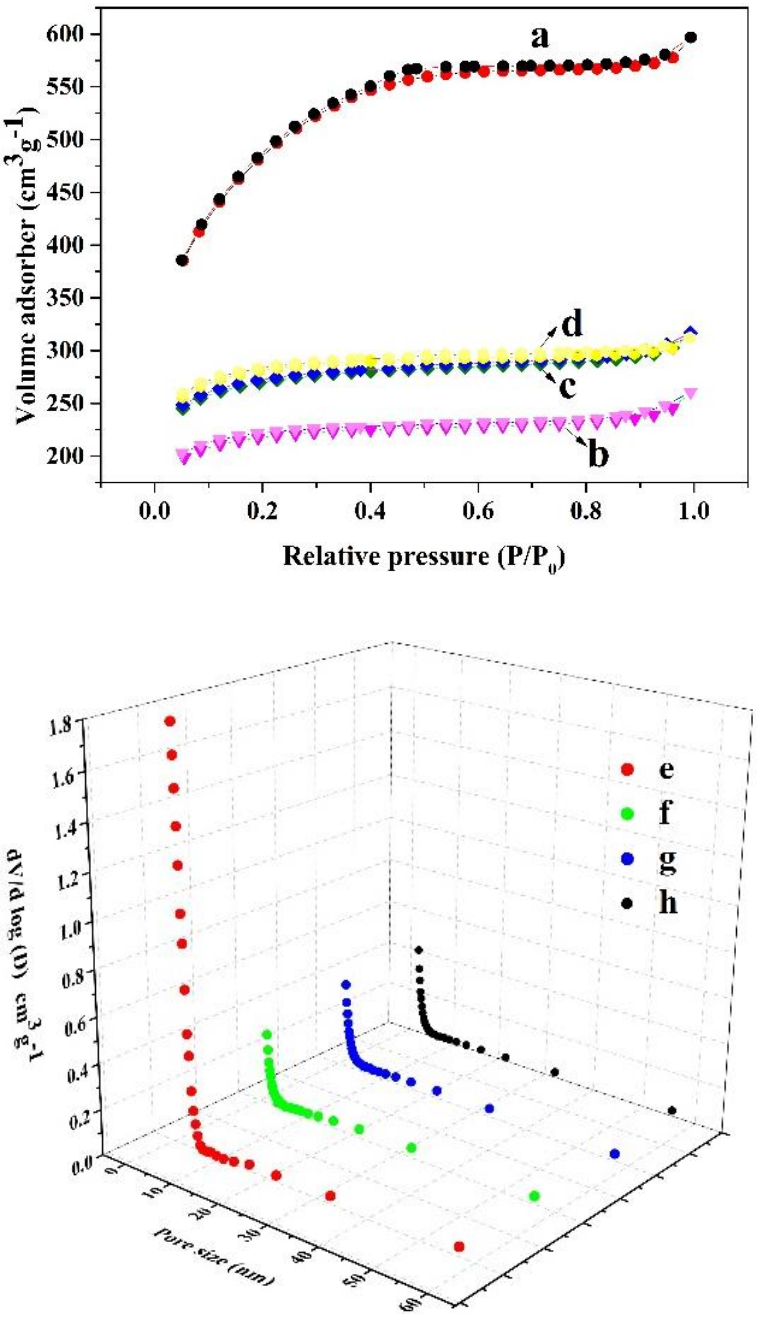

Fig. 3-3. N_2 adsorption-desorption isotherm and the pore size distribution by BJH method of C (a and e), C@CS25 (b and f), C@CS50 (c and g) and C@CS100 (d and h).

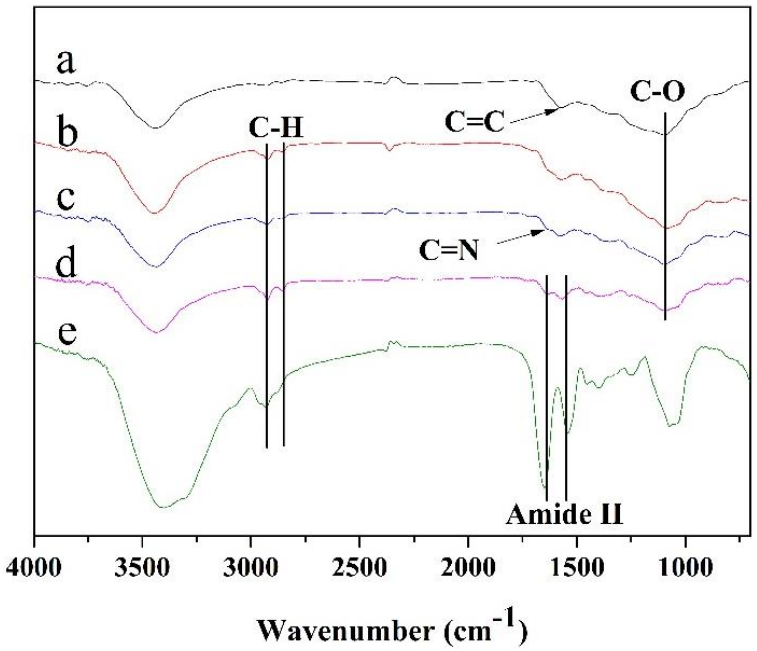

Fig. 3-4. FT-IR spectra of C (a), C@CS25 (b), C@CS25-CHO (c), C@CS25-cellulase (d) and cellulase (e).

Table 3-2. The chemical compositions of samples

Samples	Compositions		
	C/%	O/%	N/%
C	80.41	19.51	0.08
C@CS25	79.95	18.56	1.49
C@CS50	78.30	19.33	2.37
C@CS100	78.69	18.69	2.61

3.3.2 Activity assays

Fig. 3-5 shows the influence of chitosan amount on the cellulase loading capacity and activity of immobilized cellulase. 0.5 g support was added into 25 mL 4 mg/mL cellulase solution for cellulase immobilization by GA. As the chitosan addition increased from 25 to 100 mg, the cellulase immobilized amount increased and reached to 94.7, 127.1 and 167.9 mg cellulase/g support with immobilization yields of 44.5%, 64.8% and 84.5% after 12 h immobilization, respectively. Since the chitosan has a lot

of the functional groups such as hydroxyl, amino and methyl, more functional groups would be provided to immobilize enzyme as the amount of chitosan increases [43]. The activity of C@CS25- cellulase, C@CS50-cellulase and C@CS100-cellulase was 3.8, 2.9 and 1.6 IU/mg enzyme, respectively. However, a high cellulase loading capacity results in a low activity. The cellulase might be stacked during the immobilization process, which would limit its space in the hydrolysis process. Therefore, for the same quality of immobilized cellulase, when there was a stacking phenomenon or diffusion limitation, the activity would decline [44,45].

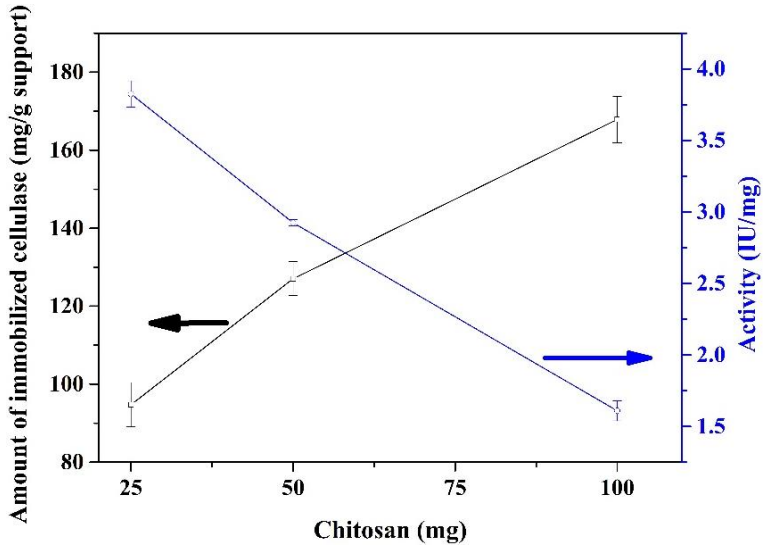

Fig. 3-5. The influence of chitosan amount on the cellulase loading capacity and activity of immobilized cellulase.

In the enzymatic hydrolysis process, the temperature and pH value of solution are important factors [46,47]. Fig. 3-6a shows the influence of pH value on the activity of free and immobilized cellulase. The optimum pH value of free and immobilized cellulase was 4 in the range of pH value = 3 to 7 at 50 °C. Compare with the free cellulase, the activity of the immobilized enzyme is not high at different pH. This is because the mobility and flexibility of cellulase are greatly limited by the multi-point binding of covalent bonds. Although C@CS50-cellulase and C@CS100-cellulase are less activity, they are more stable. This should be attributed to the multi-point covalent bond [47]. Fig. 3-6b shows the activity of free and immobilized cellulase in the range of 30 to 70 °C at pH value = 4. After immobilization, the optimum pH and temperature

did not change compared with those of free cellulase, and the value was 60 °C. C@CS25-cellulase showed the highest activity of 7.1 IU/mg enzyme at pH value = 4 and 60 °C; also, it had an activity recovery of 67% and a good stability at 50 to 70 °C. The variance of its activity values was only 0.46, whereas that of free cellulase was 1.51. This could be considered by the fact that after the porous biochar was coated with an amount of chitosan, its biocompatibility is improved while retaining a porous structure. It would be helpful to the contact of the cellulase with CMC in the enzymatic hydrolysis process [28]. At the same time, the covalent bonds would also increase their stability at a high temperature [48].

Table 3-3 is the kinetic parameter (K_m) and maximum velocity reaction (V_{max}) of free and immobilized cellulase. K_m is the characteristic physical constant of the enzyme, and determines the specificity of the enzyme and the natural substrate. The K_m value was calculated by enzymatic reaction rate and substrate concentration (1.25, 2.5, 3.75, 5, 7.5 and 10 g/L CMC solution) at pH value = 4, 60 °C for 5 min. The K_m and V_{max} were calculated by Line weaver-Burk plots though the Michaelis-Menten equation [49]. The K_m value of free and immobilized cellulase were 5.435, 7.017, 4.043 and 3.682 g/L. For free and C@CS25-cellulase, after immobilization, the increased K_m and decreased V_{max} indicated that the immobilized cellulase had a lower affinity on the substrate than the free cellulase. It should be contributed by the formed covalent bond that would lock some active sites, or bond the flexibility of enzyme. For these three immobilized enzymes, as the amount of chitosan increased, the K_m became smaller. It may be attributed to the good biocompatibility of chitosan.

Table 3-3. The K_m and V_{max} values of cellulase.

Enzyme	K_m (g/L)	V_{max} (g/(L min))
Free cellulase	5.435	0.348
C@CS25-cellulase	7.017	0.338
C@CS50-cellulase	4.043	0.172
C@CS100-cellulase	3.682	0.115

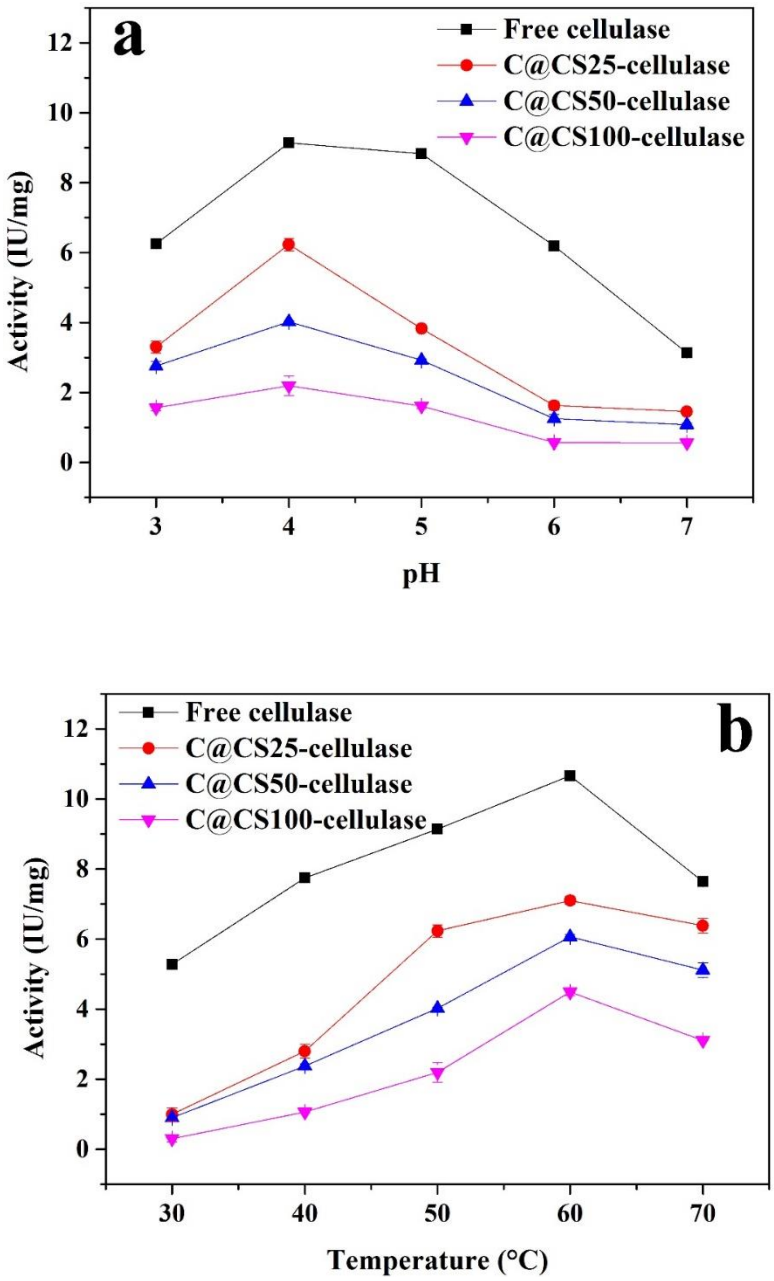

Fig. 3-6. The influence of pH value (a) and temperature (b) on activity of free and immobilized cellulase.

3.3.3 Reusability assays

Fig. 3-7 shows the reusability of immobilized cellulase. Three kinds of immobilized cellulase (containing 3 mg cellulase) were maxed with 10mL 1% CMC solution with pH value = 4, at 60 °C for 24 h. Then the mixture was separated by centrifugation at 8000 r/min for 10 min, and the immobilized cellulase was placed into a fresh CMC

solution for another hydrolysis process after washing. As shown in Fig. 7, the three kinds of immobilized cellulase showed a good reusability. The initial glucose yield of C@CS25-cellulase, C@CS50-cellulase and C@CS100-cellulase was 255.6, 227.1 and 187.9 mg glucose/g CMC, respectively; they kept 90.8%, 82.5% and 69.0% of their initial values after 10 repeated uses. For C@CS25-cellulase and C@CS50-cellulase, the yield slowly increased from cycle 2 to 5 and then gently decreased. The slight increase may conclude that there was glucose remaining on the surface of support from the previous cycle, and on the next cycle, a part of residual glucose desorbed into the fresh CMC solution under the mechanical vibration. The decrease in glucose productivity may be caused by a few part of cellulase that was inactivated with a long time hydrolysis [50]. Compared with those of C@CS25-cellulase and C@CS50-cellulase, the yield of C@CS100-cellulase declined quickly. As shows in the introduction, the glutaraldehyde-activated support could ionically adsorb enzyme by the primary amino groups before the covalent reaction. Thus, compared to the other two, in C@CS100, there should be more cellulase immobilized by ionically adsorption. And it is easy to desorb during the hydrolysis process, which leading to a decline in glucose yield.

Compared with recent reported literatures (Table 3-4), the biochar/chitosan supports possessed excellent performances for cellulase immobilization. The immobilized cellulase showed superior reusability which could reused for more than 10 times. This should be attributed to biochar/chitosan supports since they provide not only lots of active sites for enzyme immobilization, but also provide good biocompatible interface which can make the immobilized enzyme contact the substrate well.

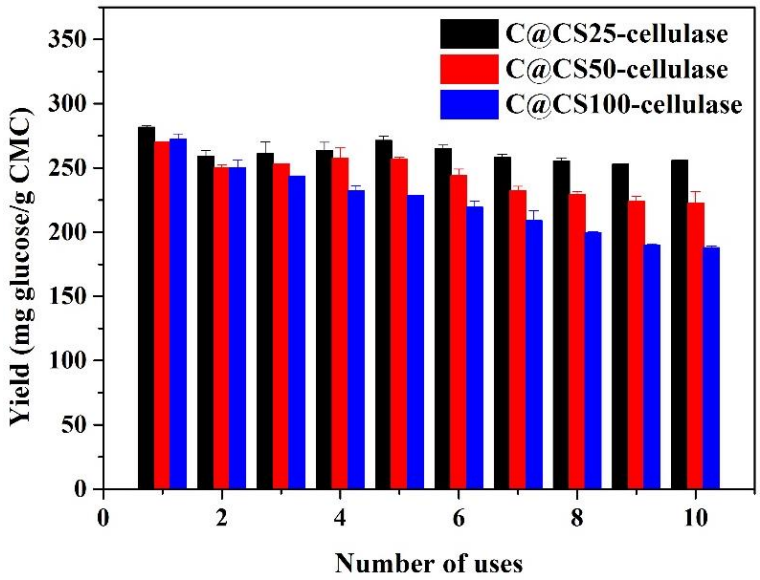

Fig. 3-7. The effect of recycles on the glucose productivity of immobilized cellulase.

Table 4. Support, immobilization method, reusability of the current work and other reports.

Support	Immobilization method	Recycle times	Residual activity	Refs
Magnetic silica nanoparticles	Absorbed	8	60%	40
Catalase@ZIF	Absorbed/embedded	13	60%	15
Non-porous MCFA/chitosan Porous MCFA/chitosan	Covalent	10 10	69.9% 75.5%	29
PS-DVB-g-PS-g-PANI	Covalent Absorbed	15 15	84% 60%	44
Magnetic silica nanoparticles	Covalent	5	75%	47
CNP/chitosan	Covalent	7	82.6%	49
Porous biochar/chitosan	Covalent	10 10 10	90.8% 82.5% 69.0%	Current work

3.4 Conclusions

Three kinds of supports for cellulose immobilization were prepared by coating different amount of chitosan on the surface of porous biochar derived from sugarcane bagasse. The amount of chitosan has influence on morphology and particle size of supports as well as cellulase loading amount, activity recovery, thermal stability and reusability of immobilized cellulase. All the three kinds of immobilized cellulase showed a good reusability. Especially for C@CS25-cellulase, it retained the morphology of porous biochar well and had an activity recovery of 67%. In addition, it remained 90.8% glucose productivity after 10 cycles. According to the results, these supports can be recommended as ideal supports to immobilize enzyme.

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Chapter 4 Preparation and characterization of magnetic polyporous biochar for cellulase immobilization by physical adsorption

4.1 Introduction

In recent years, new sustainable energy, especially bioethanol, has received wide attention. As a “green” approach, preparation of bioethanol from lignocellulosic biomass by enzymatic hydrolysis and bio-fermentation is very popular, due to the environmental friendly and low production cost [1,2]. Cellulase is a kind of complex enzyme, which consists of endoglucanase, exoglucanase and β-glucosidase. It is very useful in the lignocellulosic cellulose hydrolysis to produce glucose [3–5]. However, cellulase has good water solubility, which makes it difficult to separate from the products and cannot be recycled. Therefore, effective recovery and reuse of cellulase has received great attention.

Immobilization of bioactive materials such as DNA [6], protein [7], enzyme [8] and cell [9] on the surface of solid has been proven to be effective and feasible to recover and improve the stability and reusability of the bioactive materials. In general, the immobilization methods can be divided into physical adsorption and covalent bonding. Physical adsorption includes van der Waals force, ionic bonds, and the hydrogen bonds between solid materials and enzymes [10]. Covalent bonding means that the activated functional groups of support react with other groups (-COOH, -NH_2, -SH, etc.) [11] on the surface of the enzyme to produce a covalent bond, thereby binding the enzyme onto the support.

For the physical method, it is convenient and rarely changes the structure of enzyme, thus retaining most of the enzyme's activity [12]. Mesoporous materials have a large specific surface area, a variety of pore structures and high biocompatibility [13], which are desirable supports for enzyme immobilization. Piras [14]prepared immobilized

human lysozyme loaded into the pores of SBA-15 mesoporous silica by the immunogold staining method. Bhattacharyya [15] studied two ordered mesoporous materials that presented similar structure and texture but had different chemical composition and surface properties for lysozyme adsorption. The adsorbents showed a high lysozyme adsorption amount and provided a crucial size-selective parameter to address guest enzyme adsorption. However, for the mesoporous materials, especially silica mesoporous materials with order pore structure, how to design the size of the pore would directly affect the adsorption amount of enzyme. Simultaneously, the microenvironment of the inner of pore and the surface of adsorbent are quite different, and the mobility and flexibility of enzyme would be limited more inside the pore. Furthermore, the enzyme inside the pore is difficult or even impossible to participate in the hydrolysis reaction [16]. Therefore, developing adsorbents with large specific area, open pore structure and plenty attachment points on the surface for enzyme immobilization may relieve these negative effects.

Polyporous biochar is a kind of solid material formed from the thermochemical decomposition of lignocellulosic materials [17,18]. The main element components of biochar contain carbon, oxygen and hydrogen, and it is usually used in wastewater treatment [19] and electrode material [20]. However, the polyporous biochar-based adsorbent for cellulase adsorption has been rarely reported. On the one hand, polyporous biochar with high specific surface area and rich pore structure can provide more active sites for enzyme immobilization. On the other hand, polyporous biochar has a wide range of sources, which means it is easy to be obtained and economical. Thus, it could be an ideal adsorbent for cellulase adsorption. In addition, to facilitate recycling, combining magnetic materials could allow selective recovering from the reaction medium by applying an external magnetic field. However, there are few literatures on the preparation of magnetic polyporous biochar by calcination method that could generate a uniform magnetic particles on the surface of polyporous biochar. With the assistance of calcination method, the magnetic polyporous biochar for cellulase immobilization should be expected.

Therefore, in this work, for the adsorbent preparation, the magnetic polyporous biochar was prepared by calcination method, where the calcination time and temperature have been considered. For the cellulase adsorption, the structure of adsorbent, adsorption temperature and the adding amount of adsorbent were considered for the discussion of cellulase adsorption behavior. Finally, there was a simple evaluation of the enzymatic and reusability of the immobilized enzyme.

4.2 Experimental

4.2.1 Materials

Sugarcane bagasse was collected from Guangxi, China. Potassium hydroxide (KOH), hydrochloric acid (HCl, 35-37 wt%), ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), ethanol and carboxyl methyl cellulose sodium (CMC) were purchased from Nacalai Tesque, Inc. (Tokyo, Japan). Cellulase was bought from Meiji Seika Pharma Co., Ltd (Tokyo, Japan).

4.2.2 Preparation of polyporous biochar

The pyrolysis process for the preparation of porous biochar is widely reported, and the biomass material is usually activated by KOH [21] to obtain a larger specific surface area. Bagasse was pulverized (＜1 mm) and boiled in water at 95 °C for 8 h. After being dried at 60°C for 24 h, the bagasse was mixed with KOH and ethanol (1g: 1g: 12 mL) in a beaker at 60 °C for 5 h, and the mixture was dried at 60 °C for 12 h. Then, the dried mixture was put in a tube furnace and carbonized at 800 °C for 2 h under a N_2 protection and with a heating rate of 10 °C/min. The generated products were ground and immersed in 12.5 mL of 1.5M HCl for 2 h to remove the ash and alkali. After being washed with distilled water for several times and dried at 60 °C for 24 h, the polyporous biochar was obtained and denoted as C.

4.2.3 Preparation of magnetic polyporous biochar (C/γ-Fe_2O_3)

In order to better combine C and γ-Fe_2O_3, a calcination method has been used. At the same time, the temperature and time of calcination as well as the ratio of C, Fe^{3+} and Fe^{2+} were discussed. (a) Calcination temperature: 0.1 g C was added in 2 mL ethanol solution (containing 0.2 mmol $FeCl_3 \cdot 6H_2O$ and 0.1 mmol $FeCl_2 \cdot 4H_2O$). The mixture was dried at 70 °C for 10 min in tube furnace, and then the temperature was raised to 400~700 °C for 1 h (N_2 protection, heating rate of 10 °C/min). (b) Calcination time: the experimental procedure was similar as mentioned above. The calcination temperature was 500 °C and the calcination time was from 10 to 120 min. (c) The ratio of C, Fe^{2+} and Fe^{3+}: the mixture of C, $FeCl_3 \cdot 6H_2O$ and $FeCl_2 \cdot 4H_2O$ with three ratios of 0.1 g: 0.2 mmol: 0.1 mmol, 0.1 g: 0.4 mmol: 2 mmol, and 0.1 g: 1 mmol: 0.5 mmol were calcined at 500 °C for 1 h, respectively. The specific conditions are listed in Table 4-1.

4.2.4 Immobilization of cellulase

A certain amount of support was added in 10 mL pH value= 5 cellulase solution. The solution was placed into an air bath shaker and shaken with an oscillation speed of 120 r/min for 12 h. During this process, 0.2 mL mixture was taken out. After separation by magnet, 0.05 mL supernatant was used for the determination of residual cellulase concentration at 30 min, 1 h, 3 h, 6 h, 9 h and 12 h, respectively. The residual concentration of cellulase was determined by the Bradford protein assay method and the cellulase loading amount was calculated from the following equation:

$$\text{Immobilized amount} = C_0V_0 - C_1V_1$$

Where C_0 and V_0 is the concentration and volume of initial cellulase solution, respectively. The C_1 and V_1 is the concentration and volume of cellulase solution after immobilization, respectively.

4.2.5 Characterization

The structure and composition of samples were characterized by the X-ray diffraction

(XRD) using a PANalytical X' Pert Pro (UK) instrument. The magnetism of C/γ-Fe_2O_3 was determined by vibrating-sample (VSM, Riken Denshi Co. Ltd., Japan). The specific surface areas of samples were determined by Brunauer-Emmett-Teller (BET) nitrogen adsorption method, at 77K using ASAP 2020 analyzer (Micromeritics, USA). The morphologies of samples were analyzed by scanning electron microscope (SEM, Hitachi S-4300, Japan). The cellulase concentrations were determined by UV-vis spectrophotometer (UV-vis, U-5100, Japan).

Table 4-1 Preparation conditions of C/γ-Fe_2O_3

Number	C (g)	$FeCl_2 \cdot 4H_2O$ (mmol)	$FeCl_3 \cdot 6H_2O$ (mmol)	Temperature (°C)	Time (min)	Remarks
Temperature variable	0.1	0.1	0.2	400	60	
	0.1	0.1	0.2	500	60	
	0.1	0.1	0.2	600	60	
	0.1	0.1	0.2	700	60	
Time variable	0.1	0.1	0.2	500	10	
	0.1	0.1	0.2	500	30	
	0.1	0.1	0.2	500	60	
	0.1	0.1	0.2	500	90	
	0.1	0.1	0.2	500	120	
Feeding ratio variable	0.1	0.1	0.2	500	60	1#
	0.1	0.2	0.4	500	60	2#
	0.1	0.5	1	500	60	3#

4.2.6 Activity assay

The cellulase activity was evaluated according to IUPAC method [22]. An immobilized cellulase containing 0.2 mg of cellulase was added in 10 mL 1% CMC solution (pH value = 4). After being hydrolysed at 50 °C for 30min, the mixture was separated by a magnet for 5 min. Then, 1 mL supernatant was mixed with 2 mL DNS solution. The mixing solution was put into boiling water for 5 min. Finally, 10 mL distilled water was added to dilute the reaction solution. The amount of glucose was determined by a UV-vis spectrophotometer at 540 nm. The International Unit of cellulase activity (IU/mg cellulase) is defined as a certain amount of cellulase that hydrolyzes CMC to produce 1 μmol glucose per minute.

4.2.7 Reusability assay

For an application factor, a long enzymatic hydrolysis time would be a good method to determine the reusability of immobilized enzyme [10]. The immobilized cellulase (containing 3 mg cellulase) was distributed in 10 mL 1% CMC solution (pH value = 4) for 24 h of hydrolysis at 60 °C. After separation by a magnet, the mass of glucose in supernatant was determined by UV-vis spectrophotometer at 540 nm, and the immobilized enzyme was added into a fresh substrate solution for a new hydrolysis. This process was repeated 5 times.

$$\text{The recovery rate} = M_n / M_1 \times 100\%$$

Where M_n is the mass of glucose produced by the nth saccharfication, and the M_1 is mass of glucose produced at the first time, respectively.

4.3 Results and discussion

4.3.1 Characterization of supports

The SEM images of C and C/γ-Fe_2O_3 composites are given in Fig. 4-1 and Fig. 4-2. From the SEM image of C (Fig. 4-1a), a clearly allium-giganteum-like porous structure

[23] is found, and it has a smooth surface. After calcination, a lot of crystals grow on the surface of C/γ-Fe_2O_3 composites, indicating that γ-Fe_2O_3 has grown well on the surface of porous biochar.

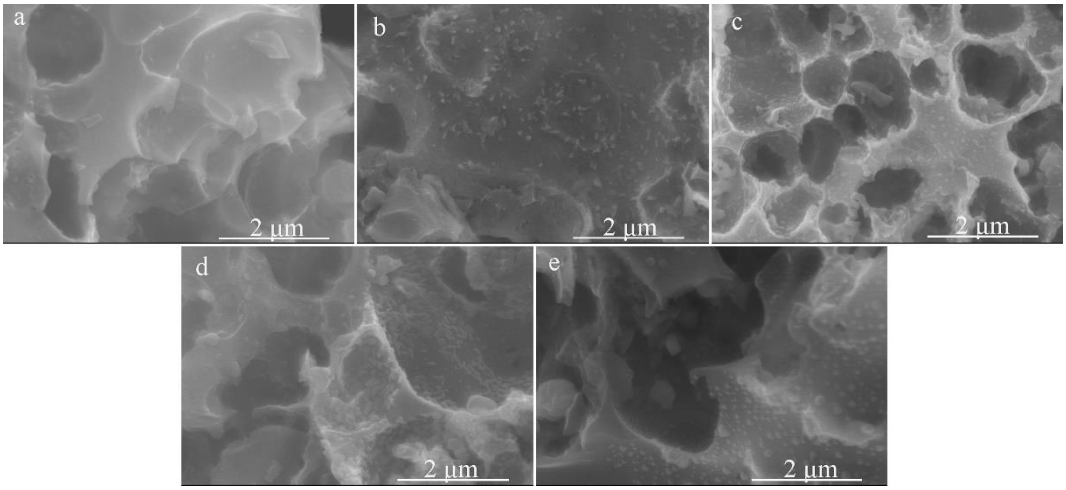

Fig. 4-1 SEM images of (a) C and C/γ-Fe_2O_3 composites calcined at (b) 400 °C, (c) 500 °C, (d) 600 °C and (e) 700 °C for 1 h.

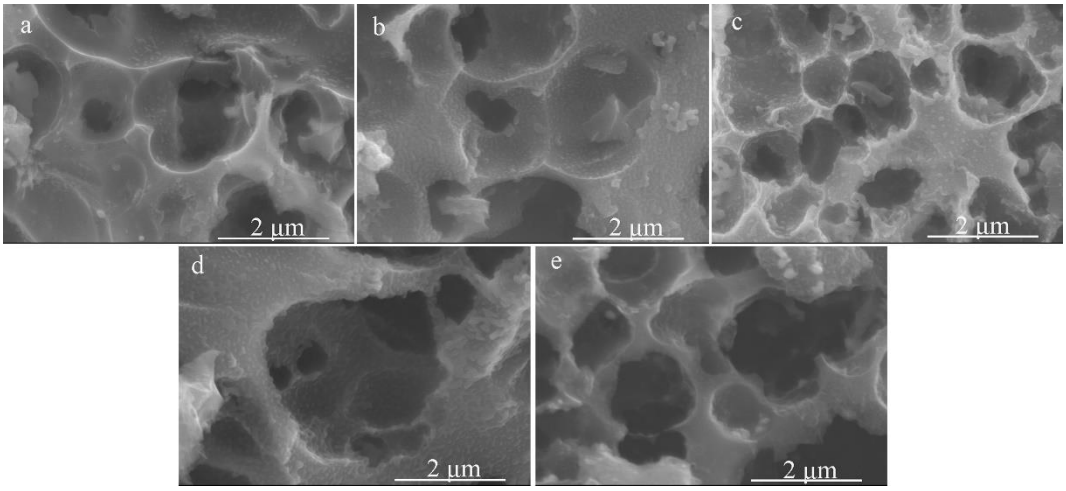

Fig. 4-2 SEM images of C/γ-Fe_2O_3 composites calcined at 500 °C for (a) 10 min, (b) 30 min, (c) 60 min, (d) 90 min and (e) 120 min.

In Fig. 4-1, the calcination temperature shows an important influence on the formation of γ-Fe_2O_3. At first, only some needle crystals are formed at 400 °C (Fig. 4-1b). As the temperature increases, the amount of crystallization also increases (Fig. 4-

1c, d). However, when the temperature reaches 700 °C, the amount of crystallization is reduced (Fig. 4-1e), which means that excessive temperature would destroy the structure of γ-Fe_2O_3. The effect of calcination time on the formation of crystals can be visually seen from Fig. 4-2, which is similar to the effect of calcination temperature. At the calcination time of 60 and 90 min, the crystals are well distributed in the polyporous biochar surface and the macroporous. However, as shown in Fig. 4-2 (e), a long time could reduce the amount of crystallization.

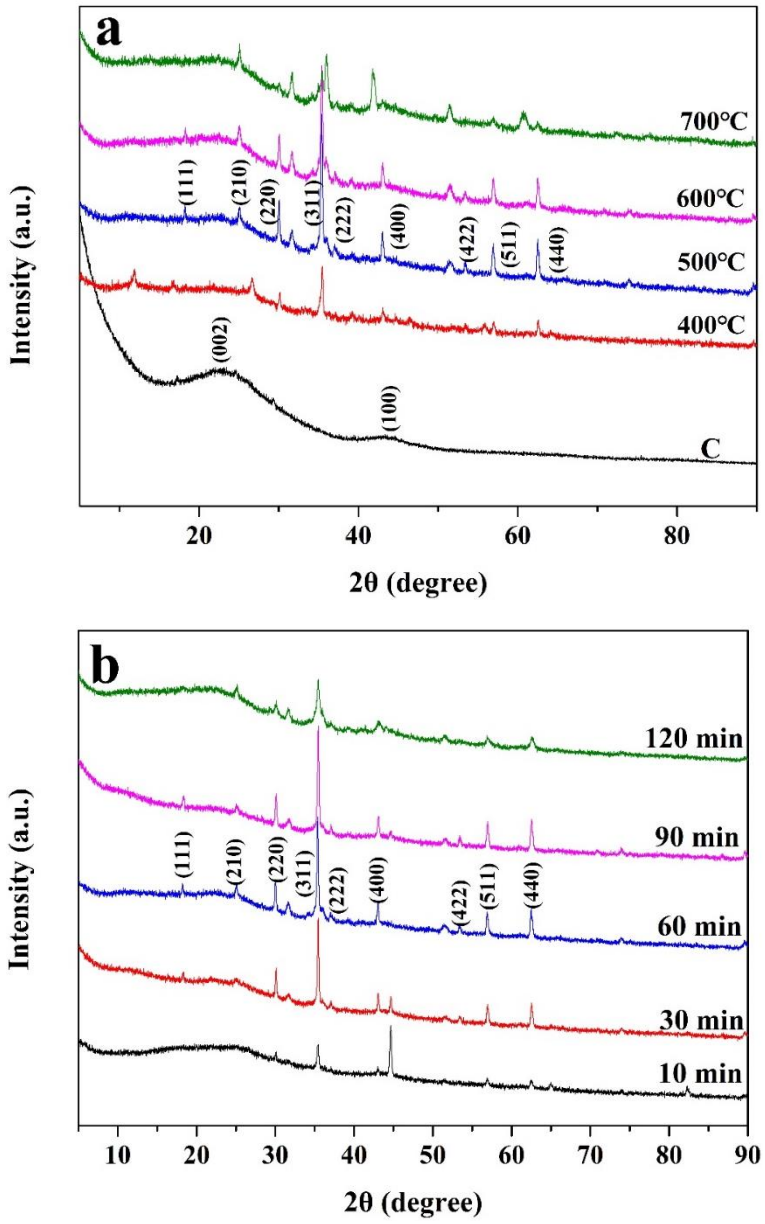

Fig. 4-3 X-ray diffraction patterns for C and C/γ-Fe_2O_3 composites with different calcination temperature (a) and time (b).

The calcination temperature and time have great influence on the formation of magnetic particles. The XRD patterns of C and C/γ-Fe_2O_3 composites with different calcination temperature are shown in Fig. 4-3 (a). For polyporous biochar, a broad diffraction peak appears at 23.1º, which is attributed to the (002) plane of graphite resulted from its amorphous nature. Meanwhile, the (100) plane of graphite is found at 43.4º (a weak diffraction peak) [24]. The results prove that bagasse has been completely converted after pyrolysis.

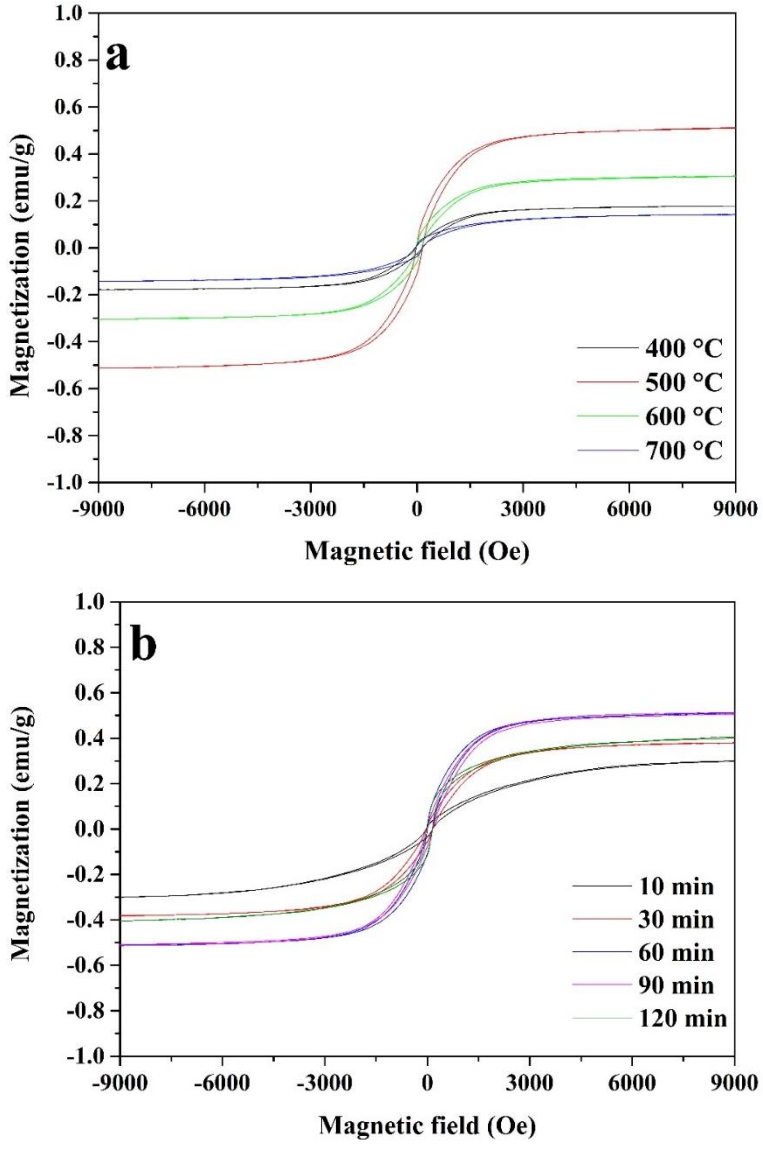

Fig. 4-4 Magnetization curves for C/γ-Fe_2O_3 composites with different calcination temperature (a) and time (b).

For C/γ-Fe_2O_3 composites calcined at 500 °C, there are 9 diffraction peaks at 2θ = 18.3º, 25.0º, 30.0º, 35.4º, 37.0º, 43.0º, 53.4º, 56.9º and 62.5º, which are assigned to the

diffraction of (111), (210), (220), (311), (222), (400), (422), (511) and (400) plans of γ-Fe_2O_3 (JCPDS card No. 39-1346)[25], respectively. When the temperature is higher than 500 °C, some diffraction peaks of γ-Fe_2O_3 become weak or even disappear because the high temperature would give rise to the partial transformation of magnetic hematite into hematite [26]. Fig. 4-3 (b) shows the XRD patterns of C/γ-Fe_2O_3 composites with different calcination time. The diffraction peaks of γ-Fe_2O_3 can be clearly found when the calcination time is 1 h. The results show that a shorter calcination time is not conducive to the formation of γ-Fe_2O_3, while a longer calcination time would destroy the structure of γ-Fe_2O_3 at 500 °C. Thus, 500 °C and 1 h are the optimum calcination conditions for the C/γ-Fe_2O_3 composites.

The magnetization curves for C/γ-Fe_2O_3 composites at room temperature are shown in Fig. 4-4. The results show that the C/γ-Fe_2O_3 composites calcined at 500 °C for 1 h has the highest saturation magnetization of 0.51 emu/g. Only a small amount of γ-Fe_2O_3 is formed with low calcination temperature and short calcination time. However, with high calcination temperature or long calcination time, the magnetic hematite would be converted into hematite. The results are consistent with the results from SEM and XRD.

4.3.2 Immobilization of cellulase by C/γ-Fe_2O_3 composites

The C/γ-Fe_2O_3 composites calcined at 500 °C for 1 h were chosen as support for cellulase adsorption. For the enzyme adsorption, the effects of adsorption temperature and amount of adsorbent were studied [27].

To investigate the effect of morphology of different adsorbents on cellulase adsorption, 50 mg C/γ-Fe_2O_3 composites was added in 10 mL 4 mg/mL cellulase solution with an air bath oscillator for at 50°C 12 h. 0.2 mL mixture was taken out for the determination of residual cellulase concentration at 30 min, 1 h, 3 h, 6 h, 9 h and 12 h, respectively. From Fig. 4-5, 1#, 2# and 3# show a similar adsorption curve for 12 h of cellulase. After 30 min of adsorption, the cellulase adsorption amount of 1#, 2# and 3# reach 108, 118 and 96 mg/g, respectively. This shows that C/γ-Fe_2O_3 composites have a good adsorption for cellulase adsorption. In addition, there are slight difference

of their adsorption curve after 9 h. 1#, 2# and 3# are close to a state of balance, while they are still slowly rising. It should be further explained in conjunction with the structure of C/γ-Fe_2O_3 composites.

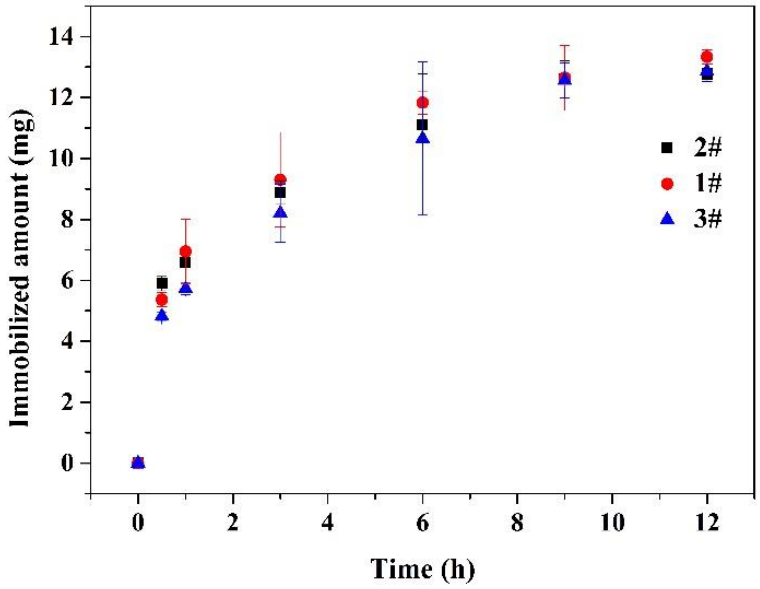

Fig. 4-5 Different adsorbents for cellulase adsorption.

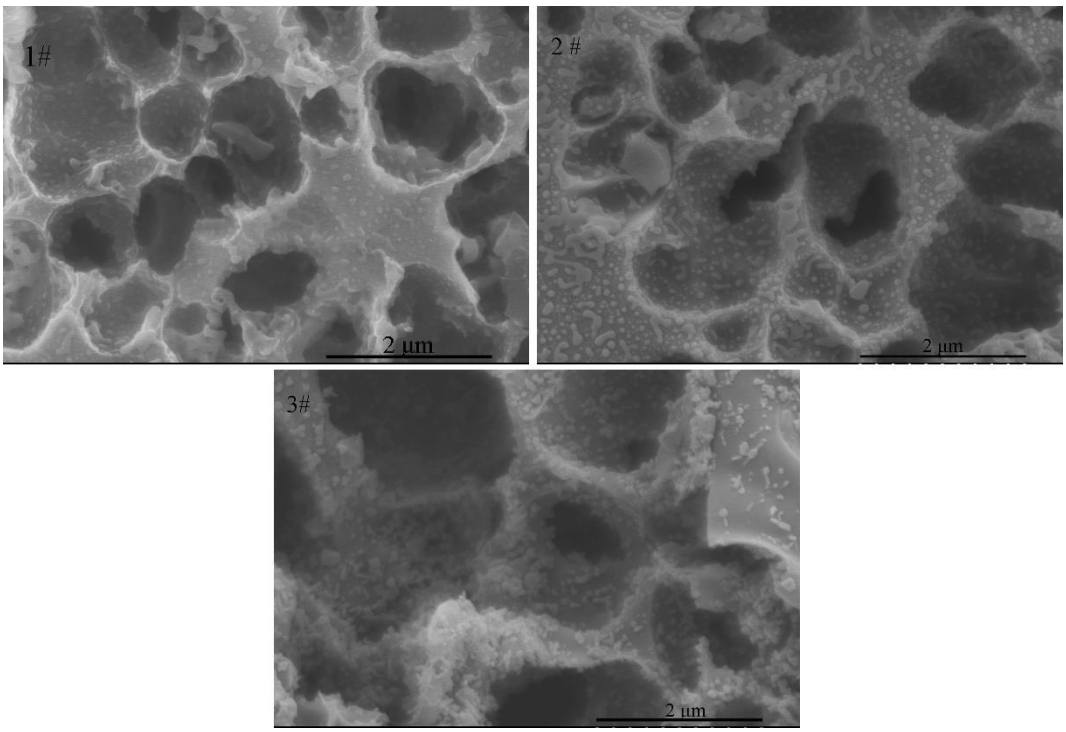

Fig. 4-6 The SEM images of 1#, 2# and 3#.

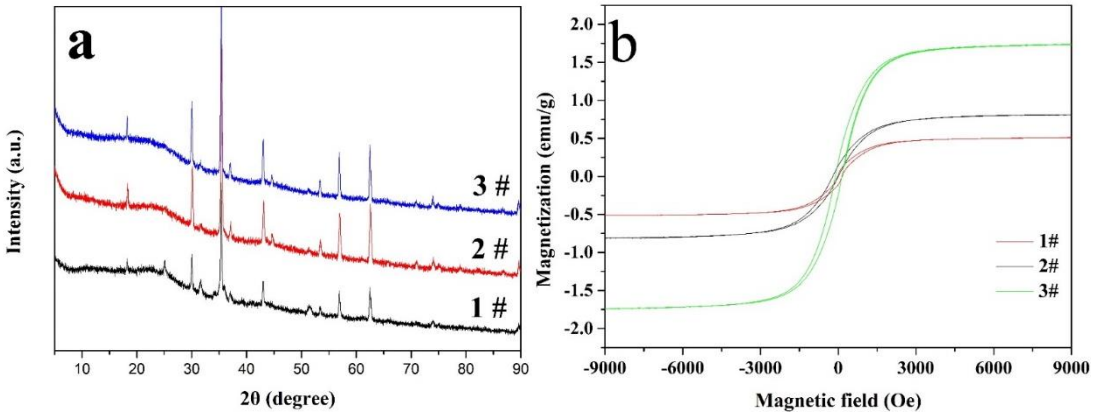

Fig. 4-7 The XRD patterns (a) and magnetization curves (b) of 1#, 2# and 3#.

As the ratio of C, Fe^{2+} and Fe^{3+} increases, the amount of crystallization also increases. The morphology of the polyporous biochar changed significantly. Especially 3#, there has been a stack of γ-Fe_2O_3 on the surface of polyporous biochar (Fig. 4-6). Meanwhile, the highest saturation magnetization increased from 0.51 to 1.74 emu/g (Fig. 4-7b). However, the X-ray diffraction patterns of 1#, 2# and 3# indicate that their crystallization structure has not been changed (Fig. 4-7a). All the composites show a combined I/IV sorption isotherm in the N_2 adsorption-desorption isotherm (Fig. 4-8), which indicates that there is a composite pore structure in the C/γ-Fe_2O_3 composites [23]. The pore size, BET surface area, total pore volume, cellulase adsorption amount and zeta potential are given in Table 4-2. Compare with 1# and 2#, the BET surface area of 3# is less than half. It is obviously caused by that the γ-Fe_2O_3 is deposited on the surface of the polyporous biochar as well as in the pores (Fig. 4-6). However, the cellulase adsorption amount of 1#, 2# and 3# are not much different, which are 266, 260, 252 mg cellulase/g adsorbent after 12 h of adsorption, respectively. This should be due to the size between pore and cellulase. Cellulase is an elongated object, and the horizontal and vertical are 12.4 and 3.7 nm [28], which is bigger than the average pore size of the adsorbents. Thus, many mesoporous structures inside the adsorbent cannot accommodate one cellulase molecule. The cellulase should be adsorbed on the surface of C/γ-Fe_2O_3 composites. Simultaneously, the zeta potentials of 1#, 2# and 3# are -37.94±5.35, -21.28±6.44 and 30.39±6.08 mV, respectively. This shows that all three materials have strong adsorption capacity. Eventually, the adsorption amounts of the

three adsorbents are not much different. However, there is still a part of cellulase that is immobilized by pore adsorption, but the process is slow. Thus, after 12 h of adsorption, the cellulase adsorption amount still rises slowly.

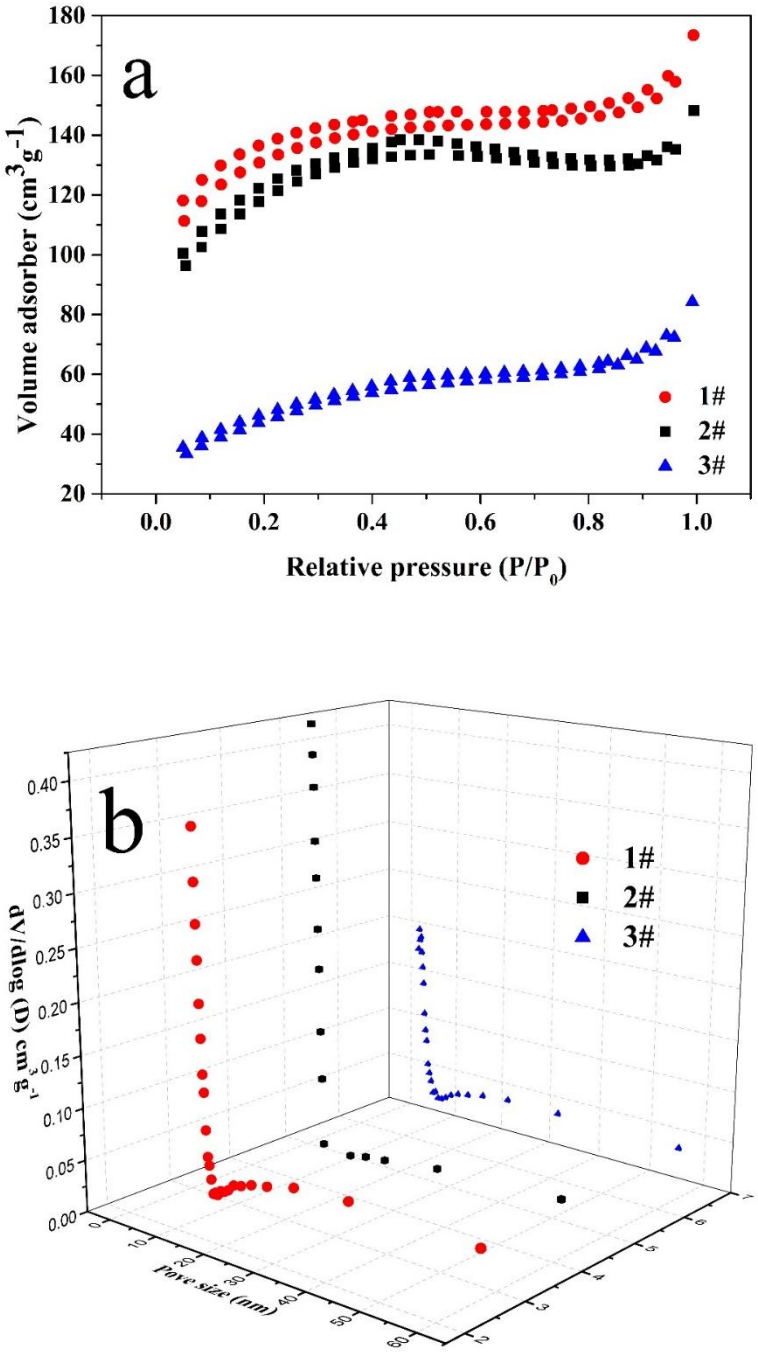

Fig. 4-8 N_2 adsorption-desorption isotherm (a) and the pore size distribution (b) by BJH method of different C/γ-Fe_2O_3 composites.

Table 4-2 The average pore size, BET surface area, total pore volume, cellulase adsorption amount and zeta potential of C/γ-Fe_2O_3 composites.

C/γ-Fe_2O_3 composites	Average pore size (nm)	BET surface area (m^2g^{-1})	Total pore volume (cm^3g^{-1})	Adsorbed amount ($mg \cdot g^{-1}$)	Zeta potential (mV)
1#	3.6	422.6	0.119	266	-37.94±5.35
2#	3.3	393.9	0.113	260	-21.28±6.44
3#	2.7	156.5	0.103	252	30.39±6.08

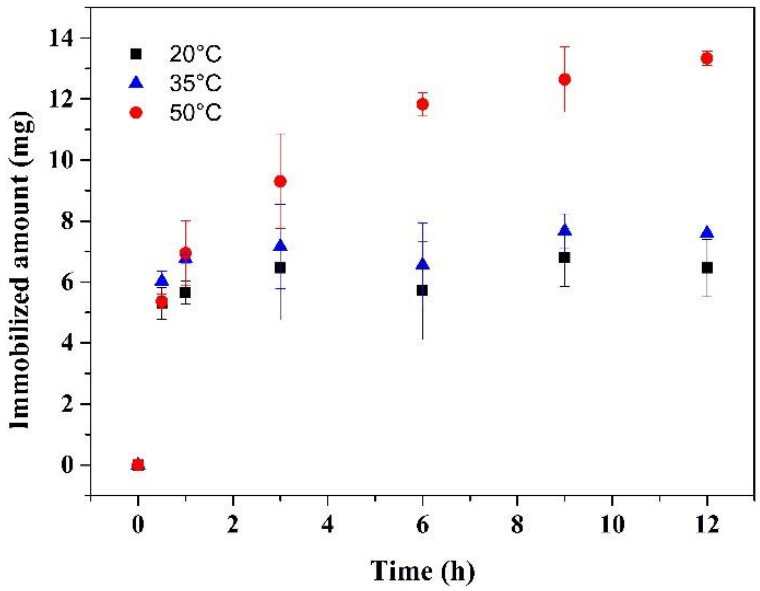

Fig. 4-9 Different temperature for cellulase adsorption.

50 mg of 1# was added in 10 mL 4mg/mL cellulase solution with an air bath oscillator for 12 h at 20, 35 and 50 °C, respectively. As given in Fig. 4-9, with the increasing temperature, the adsorption amount of cellulase is increased in the whole temperature range. According to the above analysis, a part of cellulase is adsorbed by pore adsorption. At a higher temperature, it is easy to break the initial interaction between the protein and the adsorbent surface and occurs an endothermic process [29]. Thus, the adsorption amount of cellulase would increase when the temperature increases.

However, considering excessive temperature that can cause enzyme inactivation, 50 °C is determined as the optimum adsorption temperature in this work.

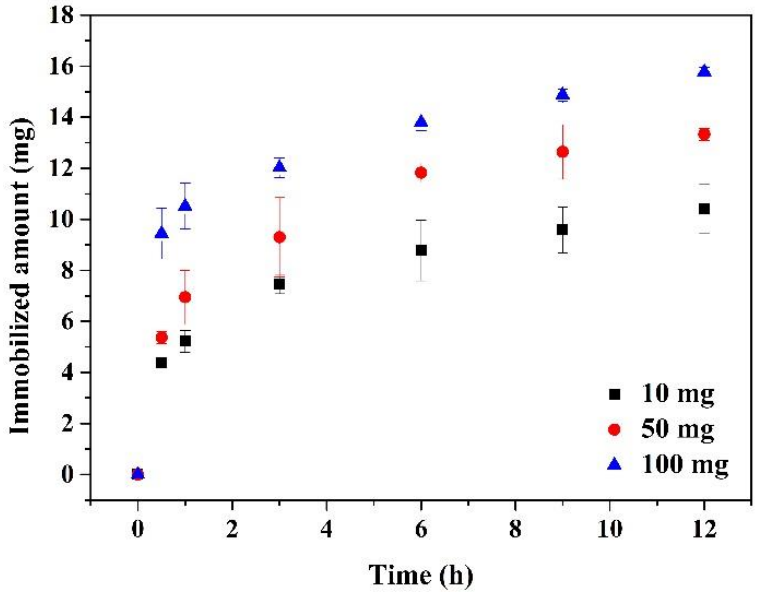

Fig. 4-10 Different adsorbent amount for cellulase adsorption.

At a certain enzyme concentration, the amount of adsorbent has a direct effect on the amount of enzyme adsorption. As shown in Fig. 4-10, 10, 50 and 100 mg of 1# were added in 10 mL 4 mg/mL cellulase solution with an air bath oscillator for 12 h at 50 °C. Obviously, the total amount of adsorption increases as the amount of adsorbent increases, and the adsorption rate of cellulase are 26.0%, 33.3% and 39.5%, respectively. When the unit is converted to mg cellulase/g adsorbent, the enzyme loading amount is 1040, 266 and 158 mg cellulase/g adsorbent, respectively. However, it can be predicted that when the adsorbent is added in an amount of 10 mg, the cellulase on the surface of the composite may be stacked. Therefore, 50 mg adding adsorbent should be the optimum adding amount in this work.

Compared with some recently reported literature (Table 4-3), the magnetic polyporous biochar possessed excellent performances for cellulase adsorption. This should be attributed to the magnetic polyporous biochar has a high specific surface area, rich pore structure and a high zeta potential which can provide more sites for enzyme immobilization. In addition, this kind of magnetic polyporous biochar is easily obtained and widely available. Therefore, it has practical value.

Table 4-3 Adsorbent, adsorbate, adsorption time and capacity of the current work and other reports.

Adsorbent	Adsorbate	Adsorption time (h)	Adsorption capacity (mg/g)	Refs
PS-DVB-g-PS-g-PANI	Cellulase	2	7.2	[30]
MNPs-APTES-Cu	Cellulase	3	164	[31]
PAA nanogel	Cellulase	2	87.3	[32]
Mesoporous silica	Cellulase	24	271.7	[28]
PVA-co-PE NFM	cellulase	4	130	[33]
Magnetic polyporous biochar	Cellulase	12	266	The current work

4.3.3 Activity assays

After being adsorbed, the structure or microenvironment of the cellulase was changed, which would change the activity of the enzyme. Fig. 4-11 is the relative activity of free cellulase, 1#, 2# and 3# immobilized cellulase. Compared with that of free cellulase, the activity of immobilized cellulase have different degrees of decline, and kept 73.6%, 66.0% and 57.5%, respectively. Cellulase was bound to the surface of adsorbent, and the mobility and flexibility of cellulase would be weaken when it is immobilized onto the surface of adsorbent, leading to a decrease in the activity of cellulase.

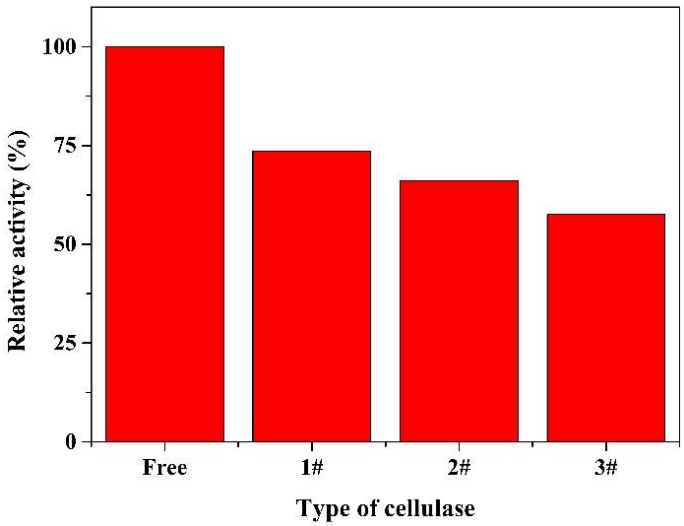

Fig. 4-11 The relative activity of types of cellulase

4.3.4 Reusability assays

To compare the relative activity of 1#, 2# and 3#, 1# was selected for reusability assays. As shown in Fig. 4-12, after the hydrolysis of CMC by 1# for 24 h, the glucose yield is 259.3 mg glucose/g CMC. However, as the number of uses increases, the glucose yield gradually decreases. This may be caused by the following reasons. The physical adsorption maybe not stronger enough, and the cellulase desorbs from the surface of polyporous biochar during the hydrolysis process. In addition, it may be caused by the end-product inhibition and protein denaturation [34].

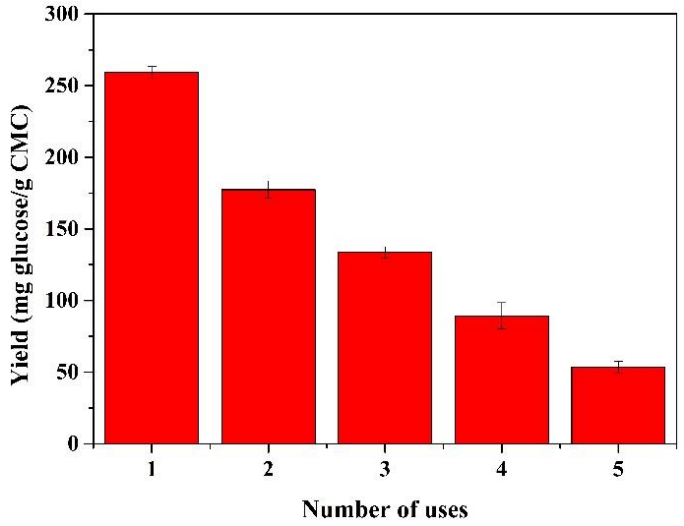

Fig. 4-12 The effect of recycles on the glucose productivity of immobilized cellulase

4.4 Conclusions

In this work, calcination method was used for the preparation of magnetic polyporous biochar. Calcination temperature and time show direct impacts on the formation of C/γ-Fe_2O_3. As the temperature increases, the amount of crystallization increases. However, higher temperature or long-term calcination would destroy the structure of γ-Fe_2O_3. The optimum condition is confirmed: calcination temperature is 500 °C and calcination time is 60 min. The large specific surface area (422.6 m^2g^{-1}) of 1# has a high zeta potential (-37.94±5.35 mV), which shows a good cellulase adsorption capacity (266 mg cellulase/g adsorbent) after 12 h of adsorption at 50 °C. Simultaneously, the immobilized cellulase retains 73.6% of the activity compared with free cellulase (at 50 °C and pH value = 4). Also, it has some reusability (maintaining 51.7% glucose yield after three uses).

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Chapter 5 Preparation of chitosan/magnetic porous biochar as support for cellulase immobilization by using glutaraldehyde

5.1 Introduction

In recent years, using biomass for bioethanol production has garnered great interest. Cellulose and hemicellulose could be hydrolyzed to reducing sugars, then the sugars are fermented into ethanol. For the process of hydrolyzing lignocellulosic materials, the use of strong acids or alkalis could increase the burden on the environment and equipment, but enzymatic hydrolysis would not. Therefore, enzymatic hydrolysis of lignocellulosic should be a greenway to produce fermentable reducing sugars [1–3]. Cellulase, a composite enzyme, mainly compose by endo-l, 4-β-D-glucanase, exo-l, 4-β-D-glucanase, and β-glucosidase. Its classification is based on attacking the depolymerization stage of the substrate. Endoglucanases randomly hydrolyze the glycosidic bonds in the amorphous regions of cellulose to produce oligomers with several degrees of polymerization. Then, exoglucanase hydrolyzes the β-1, 4-glycosidic bond of the oligomer to produce cellobiose. Finally, cellobiose is degraded to glucose by β-glucosidase [4]. However, some factors limit the application of free cellulases, such as changes in pH, temperature and ionic strength, product inhibition, and difficulty in recovering from the reaction medium. Therefore, it is meaningful to improve the stability and reusability of cellulase [5,6]. Several methods could be used for enhancing the stability of the enzyme, such as protein engineering, chemical modification, and immobilization [7,8]. Among them, immobilization has more advantages in the isomerization of the enzymatic reactions and reusability [9].

Enzyme immobilization on a solid support can improve its stability and makes it easy to recover from the medium and soluble substrate, which has been proven [10,11]. In recent years, various types of solid supports are used for enzyme immobilization, such

as natural clays [12,13], gels [14,15], and porous materials. Usually, natural materials have good biocompatibility and rich functional groups, but the low surface area limits its application. Therefore, the porous materials have a large specific surface area could provide more space for enzyme immobilization. Mesoporous silica [16], metal-organic framework materials [17], and zeolites [18] are popular support in enzyme immobilization. However, among them, the preparations are complicated and normally require precision which would increase the costs.

Biochar, a solid porous particle obtained by pyrolysis of biomass in the absence of oxygen [19], is popular in soil amendment [20], wastewater treatment [21], and electrode material [22]. Simultaneously, it is emerging as promising support to immobilize enzymes. Porous biochar has a high specific surface area (~1600m^2/g), and different types of pore structures [23,24]. Compared with other materials (mesoporous silica, zeolite, graphene, and metal-organic framework), porous biochar has the advantages of abundant sources, simple preparation method, and cheap. However, the insufficient of reactive groups and hydrophilic groups, and inconvenient recovery limit its application in the immobilization of hydrophilic enzymes.

In order to conveniently and quickly recycle and reuse enzymes, the magnetic base material has been paid much attention because it can be easily separated from the reaction system just by applying a magnet [25–28]. Co-precipitation and hydrothermal methods are commonly used to prepare magnetic-based materials. However, it is difficult to use the two methods to prepare the magnetic base biochar because there are few functional groups on the surface of the activated porous biochar. If the iron ions are firstly dispersed and attached to the biochar, the magnetic particles can be uniformly grown in the biochar after calcination. This should be a good strategy to prepare magnetic biochar. In order to improve the biocompatibility of magnetic-based biochar, chitosan is usually used to modify the supports because it has good hydrophilic, biocompatible, and non-toxicity. In addition, chitosan can provide amino groups to covalently bind with the enzymes [29–31].

Covalent attachment is a very convenient method for enzymatic immobilization. It

has been proven to be more efficient and can provide more stable biocatalysis [32]. Glutaraldehyde should be the most widely used as a cross-linking agent because it is facile, efficient, and can improve the stability of enzyme by multipoint or multisubunit immobilization. The support with primary amino groups can be activated by glutaraldehyde. Then, the glutaraldehyde-activated support reacts with the primary amino groups of enzymes. For immobilized cellulase, the glutaraldehyde activated carrier may be sterically hindered because of its spacer arms. However, it can be considered as hetero-functional support that can provide chemical reaction groups and anion exchange, and gives the highest reactivity with the amino groups of the protein [33]. Epoxy and di-vinyl-sulfone (DVS) activated supports are also popular for enzyme immobilization by multipoint covalent attachment, but they have some limitations. Such as the low reactivity of epoxy-activated support [33], and the low activity recovery of DVS- activated support [34]. In addition, dithiocarbamate (DTC) is of great interest as a new functional group for covalent immobilization. The amination carrier is firstly modified with carbon disulfide to generate a DTC group and then covalently bonded to the amine group on the surface of the enzyme [35,36]. Though it is an effective method and the DTC has a shorter spacer arm, the method still needs further discussion and the carbon disulfide is an enzyme inhibitor. Therefore, glutaraldehyde was selected as the covalent agent for cellulase immobilization in this work.

Based on these backgrounds, the main objective of this work: We aim to use porous biochar (obtained from agricultural waste sugarcane bagasse) as a basis to obtain chitosan/magnetic porous biochar after magnetization and functionalization. And then it was used as a support for the cellulase immobilization via glutaraldehyde. The structure and morphology of the support have been characterized, and the enzymatic properties of free enzyme and immobilized enzyme were evaluated in hydrolyzed carboxyl methyl cellulose sodium, including optimum pH and temperature, kinetic parameters, and reusability.

5.2 Experimental

5.2.1 Materials

Sugarcane bagasse of cane was produced in Guangxi, China. Potassium hydroxide (KOH), hydrochloric acid (HCl, 35-37 wt%), ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), chitosan (CS), acetic acid (HAc), sodium acetate (NaAc), glutaraldehyde (GA, 25%, v/v) and carboxyl methyl cellulose sodium (CMC) were purchased from Nacalai Tesque, Inc. (Tokyo, Japan). Cellulase (pale yellow powder) was bought from Meiji Seika Pharma Co., Ltd (Tokyo, Japan).

5.2.2 Support preparation

First of all, the porous biochar was prepared from sugarcane sugarcane bagasse by pyrolysis with KOH activation [37]. After boiled processing at 95 ºC for 8h, impurities on the surface of sugarcane bagasse were removed. The dry pretreated sugarcane bagasse was mixed with KOH and ethanol, and the ratio is 1 g: 1 g: 12 mL. The mixture was thoroughly mixed (500 r/min, 60 ºC for 5 h) and then dried (60 ºC for 12 h). And it was pyrolyzed in a tube furnace with nitrogen protection at 800 ºC for 2 h (heating rate: 10 ºC/min). After grinding and soaking in 1.5 M HCl solution to remove ash and alkali, the porous biochar was washed with distilled water and dried (80 ºC for 24 h), and it was denoted as C.

In order to improve the combination of porous biochar and magnetic, a calcination method has been used [38]. 0.1 g porous biochar, 0.2 mmol $FeCl_3 \cdot 6H_2O$, and 0.1 mmol $FeCl_2 \cdot 4H_2O$ were dispersed in 2 mL ethanol solution. The mixture was calcined in a tube furnace at 500 ºC for 1h under nitrogen protection (heating rate: 10 ºC/min). Finally, magnetic porous biochar was obtained and denoted as C/γ-Fe_2O_3.

Before cellulase immobilization, it is very effective and convenient to modify biochar with chitosan to improve its biocompatibility and increase its surface functional groups. 0.5 g C/γ-Fe_2O_3 was added into 25 mL of 1% (v/v) acetic acid solution (containing 50 mg chitosan) with a strong stirring at room temperature for 30 min, and

then, it was mixed with 25 mL of 1M NaOH solution. The products were recovered by a magnetic and washed with distilled water for 5 times. And it was denoted as C/γ-Fe_2O_3@CS.

5.2.3 Cellulase immobilization

In this part, C/γ-Fe_2O_3@CS was activated by glutaraldehyde. The support obtained above was dispersed in 25 mL of 2.5% (v/v, dissolved in distilled water, pH7) glutaraldehyde solution at room temperature for 2.5 h. After that, the activated support was washed with distilled water and 0.1 M HAc-NaAc buffer solution (pH=5) for 3 times.

In the cellulase immobilization process, the activated support was put in 25 mL of 4 mg mL^{-1} cellulase solution (400 mg of cellulase powder was dissolved in 100 mL of 0.1 M pH=5 HAc-NaAc buffer solution at room temperature) with a low stirring at room temperature for 12 h. The products were washed with 0.1 M pH=5 of HAc-NaAc buffer solution for three times and recovered by a magnetic. The immobilized cellulase was stored at 4 ºC, and the supernatant was used to determine the concentration of residual cellulase by the Bradford protein assay method [39]. The cellulase immobilization amount and yield were calculated by the following equation:

$$\text{Cellulase immobilization amount} = C_0V_0 - C_1V_1$$

Where C_0, C_1, V_0, and V_1 are the concentration and volume before and after cellulase immobilization, respectively.

5.2.4 Characterizations

The X-ray diffraction (XRD) was used to determine the structure and composition of samples. And the scanning electron microscope (SEM, Hitachi S-4300, Japan) analyzed the morphologies of particles. The magnetism of samples was characterized by vibrating-sample (VSM, Riken Denshi Co. Ltd., Japan). The Brunauer-Emmett-Teller (BET, Micromeritics, USA) determined the average pore size, BET surface area, and total pore volume of samples by nitrogen adsorption method at 77 K. The chemical

structures of samples were confirmed by Fourier transform infrared spectroscopy (FT-IR, iN10MX, Thermo Fischer Scientific, USA). The amount of cellulase and reducing sugar were determined by UV spectrophotometer (UV-vis, U-5100, Japan).

5.2.5 Activity assay

The enzyme activity was determined by the IUPAC method [40]. The steps of activity assay were as follows: for the free cellulase, 0.5 mL cellulase solution (0.02 $mg \cdot mL^{-1}$, dissolved in 0.1 M HAc-NaAc buffer) was reacted with 0.5 mL of 1% (m/v) CMC for 30 min; for the immobilized cellulase, the samples (containing 0.2 mg of cellulase) were dispersed in 10 mL of 0.1 M buffer solution and mixed with 10 mL of 1% (m/v) CMC solution (both of them were preheated) for 30 min. The supernatant was used for measuring the amount of reducing sugars by the dinitrosalicylic acid (DNS) colorimetric method. The cellulase activity (IU/mg cellulase) is defined as that the CMC was hydrolyzed by the amount of cellulase to produce μ mole reducing sugars per minute. To evaluate the effects of pH and temperature on cellulase activity, the hydrolysis reactions were carried out at different pH (3.0 to 7.0 at 50 ºC) and temperatures (30 to 70 ºC at pH 4).

5.2.6 Kinetic assay

This assay was performed by measuring the glucose produced by cellulase hydrolyzing different concentrations of substrates at the optimum pH and temperature for 5 min. The samples (containing 0.2 mg of cellulase) were dispersed in 10 mL of 0.1 M pH=4 HAc-NaAc buffer solution and mixed with 10 mL different concentrations of CMC solution (5, 7.5, 10, 12.5, and 15 $g \cdot L^{-1}$ 0.1 M pH=4 HAc-NaAc buffer solution) at 60 ºC for 5 min. The Michaelis–Menten constant (K_m) and maximum reaction velocity (V_{max}) were determined by Lineweaver-Burk plot.

5.2.7 Reusability assay

Base on the viewpoint of practical applications, a longer hydrolysis time was used to

determine the reusability of immobilized cellulase [5]. The immobilized cellulase that containing 3 mg cellulase was mixed with 10 mL 1% (m/v) CMC solution (pH=4) for 24 h at 60 ºC. Then, the immobilized cellulase was recycled by a magnetic and added to a fresh CMC solution for another cycle. The reusability was evaluated by the production of reducing sugars from each cycle and the reusability assay was repeated 10 times.

5.3 Results and discussion

5.3.1 Characterization of supports and cellulases

Fig. 5-1 shows the XRD patterns of biochar (black curve), biochar/γ-Fe_2O_3 (red curve), biochar/γ-Fe_2O_3@chitosan (blue curve), and immobilized cellulase (pink curve). There are two broad diffraction peaks that appear at 23.1º and 43.4º in the pattern of a. And they should be the (002) and (100) plane of graphite [41]. From the patterns of biochar/γ-Fe_2O_3, all the diffraction peaks can be indexed to γ-Fe_2O_3 according to the JCPDS no. 39-1346 [42], which indicates that the γ-Fe_2O_3 has grown on the surface of porous biochar. After coating chitosan and immobilizing cellulase, the crystal of γ-Fe_2O_3 has not change resulting from blue curve and pink curve.

The SEM images of porous biochar and its magnetic composites are given in Fig. 5-2. A porous structure is found in porous biochar (d), which has a smooth surface. After mixing with the iron and calcination, there are a lot of crystals grown on the surface of porous biochar, indicating that the porous biochar can be well combined with magnetic particles by calcination. From c and f, a thin layer is covered on the surface of biochar and magnetic particles after chitosan modification, and the macropore structure of porous biochar is well maintained, which is helpful for the substrate to diffuse into the pores so as to improve the enzymatic performances.

Fig. 5-3 shows the Magnetization curves for biochar/γ-Fe_2O_3 and biochar/γ-Fe_2O_3@chitosan at room temperature. It can be seen that the saturation magnetization of biochar/γ-Fe_2O_3 is 0.81 emu/g. however, the saturation magnetization of biochar/γ-

Fe_2O_3@chitosan decrease to 0.67 emu/g. It should be caused by the non-magnetic chitosan coating on the biochar/γ-Fe_2O_3 surface and the weight conversion of the coating. It can be clearly seen from the photos in Fig. 5-3 that most of biochar/γ-Fe_2O_3@chitosan can be easily recovered by a magnet.

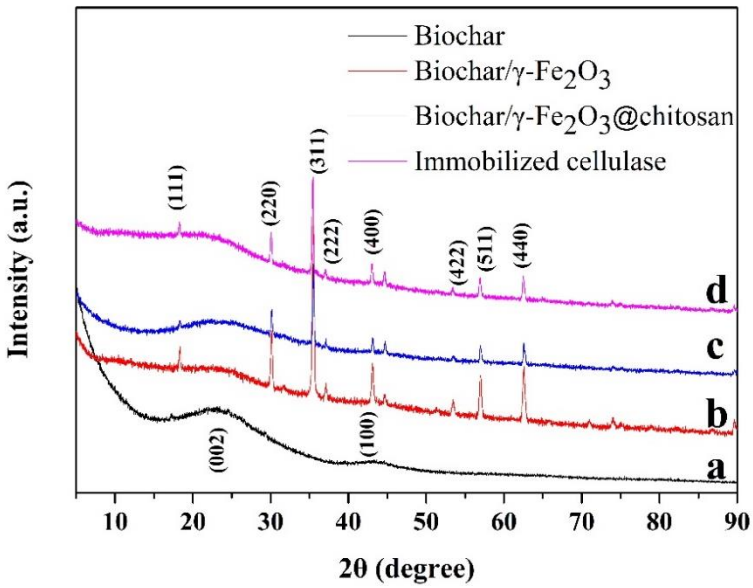

Fig. 5-1 XRD patterns of biochar (black curve), biochar/γ-Fe_2O_3 (red curve), biochar/γ-Fe_2O_3@chitosan (blue curve), and immobilized cellulase (pink curve).

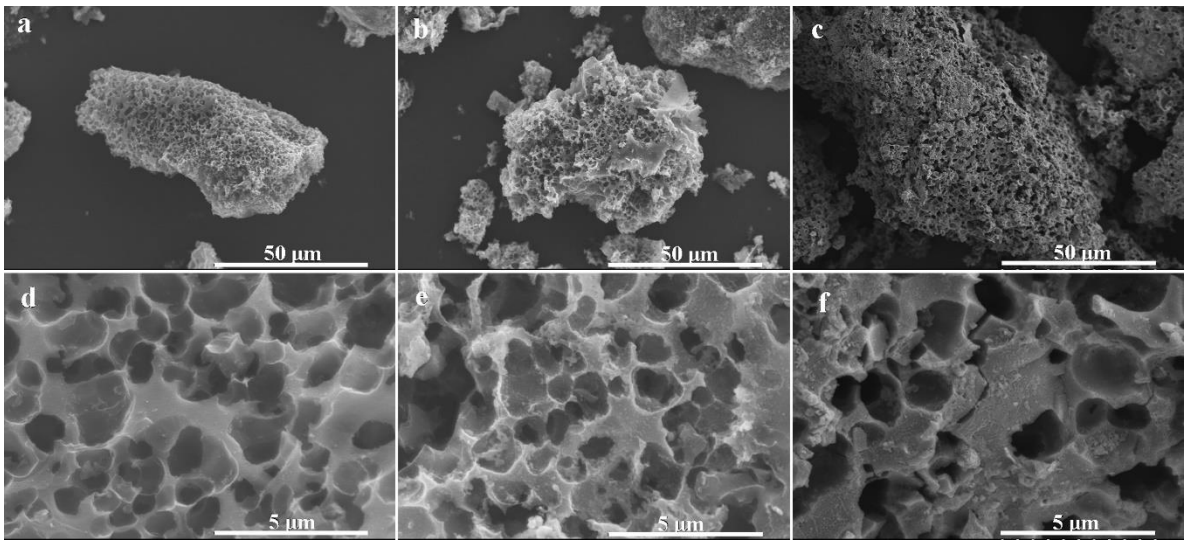

Fig. 5-2 SEM images of (a, d) biochar, (b, e) biochar/γ-Fe_2O_3 and (c, f) biochar/γ-Fe_2O_3@chitosan. Images d, e, and f are magnified images at the center of the materials in a, b, and c, respectively.

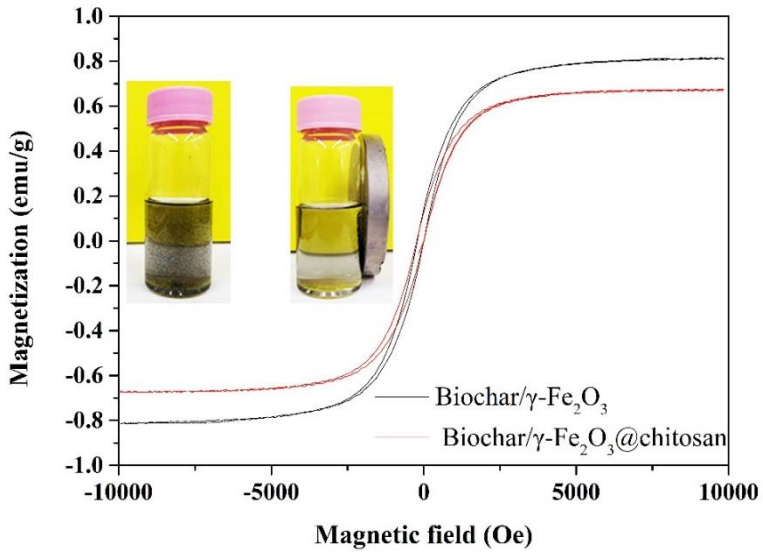

Fig. 5-3 VSM magnetization curves of biochar/γ-Fe_2O_3 (black curve) and biochar/γ-Fe_2O_3@chitosan (red curve). The insets show the state of biochar/γ-Fe_2O_3@chitosan before and after being recovered by the magnet.

Fig. 5-4 is the N_2 adsorption-desorption isotherm (a) and the pore size distribution (b) by BJH method of porous biochar, biochar/γ-Fe_2O_3, and biochar/γ-Fe_2O_3@chitosan. The nitrogen adsorption-desorption isotherms of the samples exhibit a combination of type I and IV shaped according to IUPAC classification, which explains that the samples contain both micropores and mesopores [38]. However, the N_2 adsorption capacity of biochar has decreased after modification with γ-Fe_2O_3 and chitosan. From table 1, the average pore size, BET surface area, and total pore volume of porous biochar, biochar/γ-Fe_2O_3, and biochar/γ-Fe_2O_3@chitosan are given. After modification of the magnetic material and chitosan, the BET surface area of the support is reduced from 1595.7 to 271.6 m^2 g^{-1}. This is because, in the process of modifying the support, the modification coats the surface of the porous biochar and the inner wall of the pores, which could block a part of the mesopores of the biochar. After modification, the average pore size of the sample increased. This is because iron ions adhere to the biochar and then the magnetic iron oxide crystals grow after calcination. During this process, some of the small pores are blocked, so the average pore size of biochar/γ-Fe_2O_3 becomes larger than that of biochar. After chitosan modification, flocculent

chitosan is covered on the surface of the sample, causing some small pores to be blocked, leading to an increase in average pore size.

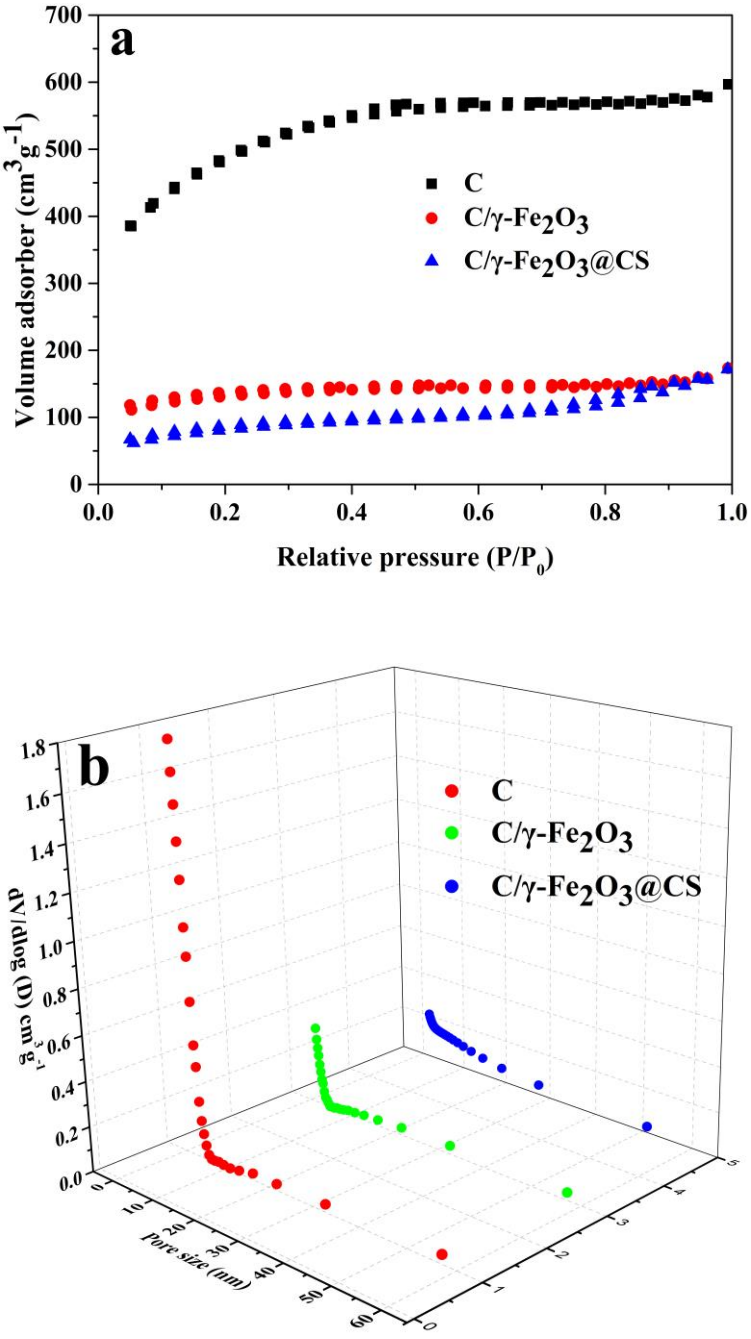

Fig. 5-4 (a) N_2 adsorption-desorption isotherm and the (b) pore size distribution by BJH method of porous biochar (black square), biochar/γ-Fe_2O_3 (red ball), and biochar/γ-Fe_2O_3@chitosan (blue triangle).

Table 5-1 The average pore size, BET surface area, and total pore volume of biochar, biochar/γ-Fe_2O_3, and biochar/γ-Fe_2O_3@chitosan.

Samples	Average pore size (nm)	BET surface area (m^2g^{-1})	Total pore volume (cm^3g^{-1})
Biochar	2.6	1595.7	0.923
Biochar/γ-Fe_2O_3	3.6	421.3	0.119
Biochar/γ-Fe_2O_3@chitosan	3.8	271.6	0.208

The chemical functional group of the samples: biochar (black curve), biochar/γ-Fe_2O_3 (red curve), biochar/γ-Fe_2O_3@chitosan (green curve), immobilized cellulase (pink curve), and free cellulase (blue cure) were determined by FT-IR. As shown in Fig. 5-5, the C-O stretching vibration of porous biochar was found at 1100 cm^{-1} [39]. For biochar/γ-Fe_2O_3@chitosan, the methylene stretching vibrations at 2924 and 2856 cm^{-1} were attributed to the chitosan layer [40]. Besides, the amide II stretching vibrations of cellulase at 1648 and 1560 cm^{-1} were found in the immobilized enzyme, which suggests that the cellulase was successfully immobilized onto the support.

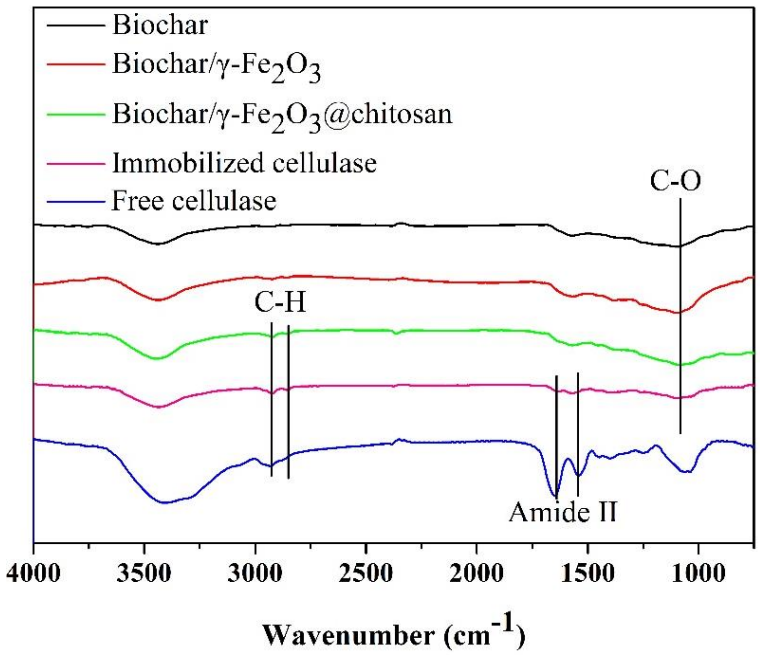

Fig. 5-5 FT-IR of biochar (black curve), biochar/γ-Fe_2O_3 (red curve), biochar/γ-Fe_2O_3@CS (green curve), immobilized (pink curve) and free cellulase (blue cure).

5.3.2 Effect of pH and temperature on cellulase activity

According to the Bradford protein assay method and calculating by the equation, the cellulase immobilization amount and rate is 80.5 mg cellulase/g support and 40.25%, respectively. In this process, the multi-point or multi-subunit immobilization is a slow process that the cellulase could be adsorbed onto the glutaraldehyde-activated support before it. After three washings with buffer, the adsorbed cellulase is desorbed from the support. Moreover, the acidic condition is not ideal for multi-point immobilization [31].

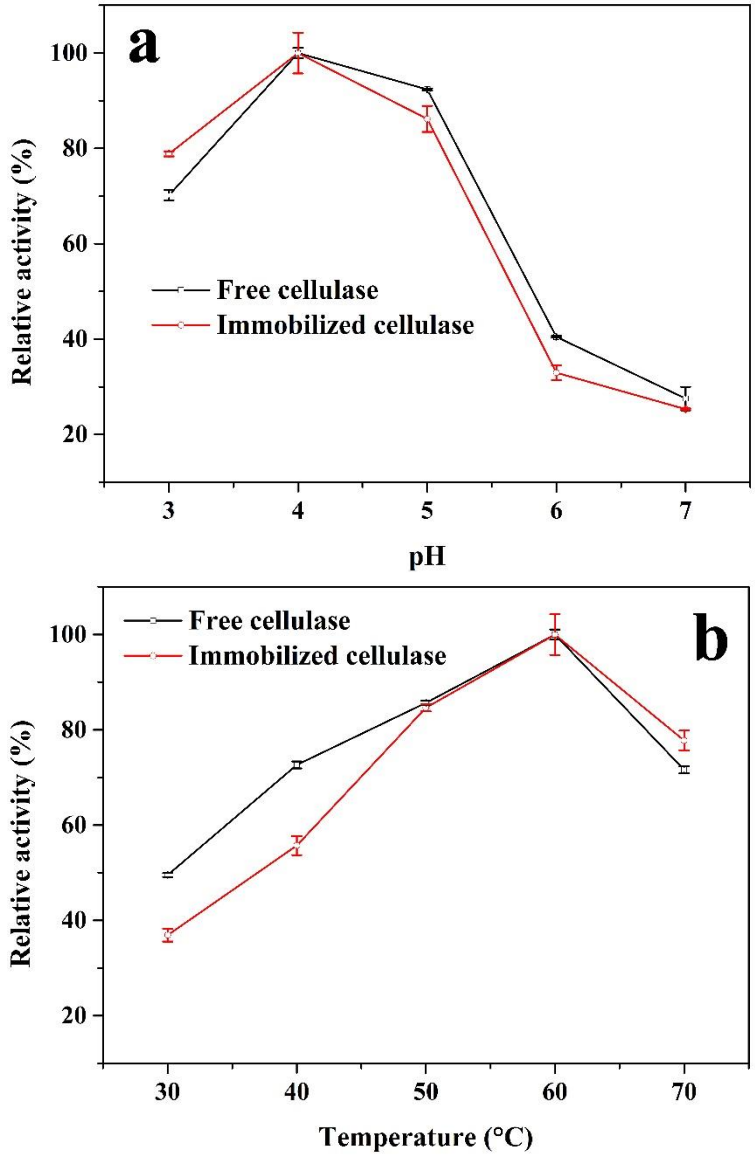

Fig. 5-6. Influences of (a) pH and (b) temperature on the relative activity of free cellulase (black curve) and immobilized cellulase (red curve).

After immobilization, the structure of cellulase may be altered which would change the accessibility of the active site, stability, and specificity [41]. Therefore, it is necessary to investigate the influence activity by pH and temperature between free and immobilized enzymes. Fig. 5-6a shows that the relative activity of free and immobilized cellulase has a similar trend and the optimal pH is 4. It could be suggesting that there are few alterations of the cellulase after immobilization.

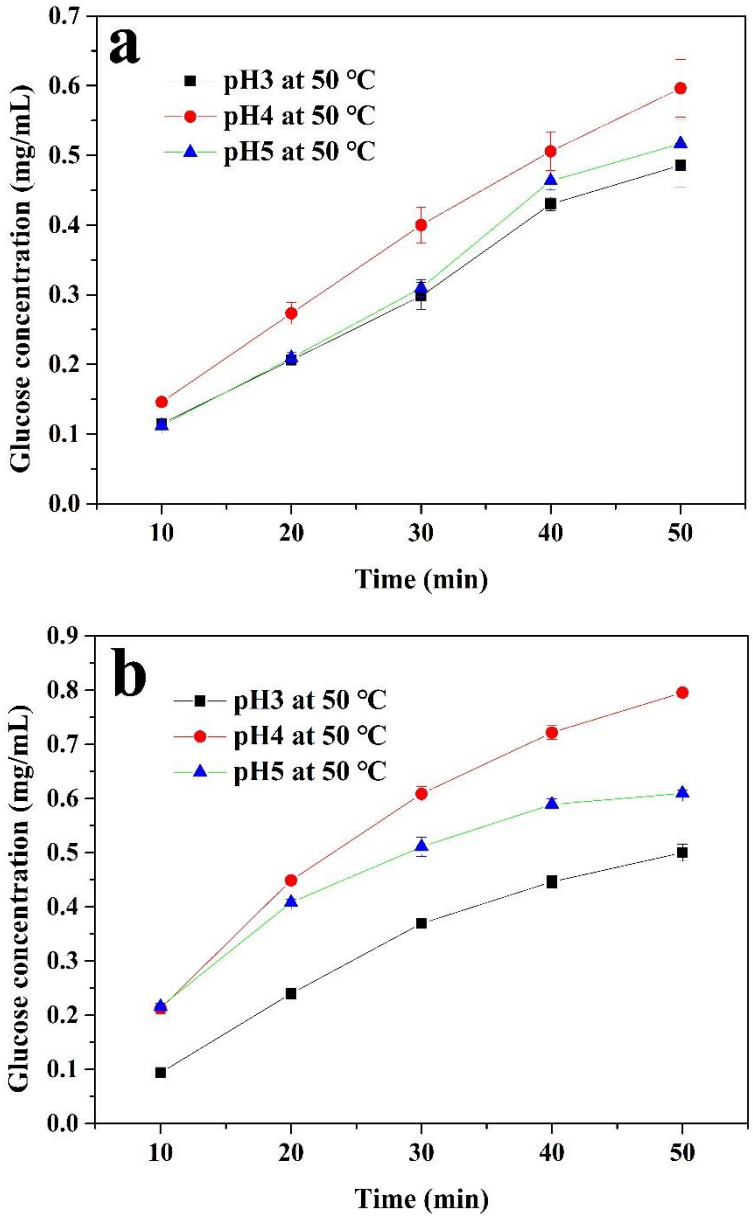

Fig. 5-7 The glucose produced from (a) immobilized cellulase and (b) free cellulase by hydrolyzing 1% CMC solution at 50 ºC with different pH values for 50 min.

In addition, the immobilized cellulase shows higher relative activity than the free cellulase at pH 3. Under acidic conditions, protonation of chitosan is easily and it is possible to interact with more CMC [40]. It means that during the hydrolysis process, the CMC concentration around the immobilized enzyme could be higher than that of the free one at a lower pH, which could promote the hydrolysis process. The influence of relative activity on the thermal characteristics of both enzymes was determined in the range from 30 to 70 ºC at pH 4. The result shows in Fig. 5-6b and the highest activity appears at 60 ºC. At 30 and 40 ºC, the relative activity of the immobilized cellulase is slightly lower than that of the free enzyme. The fluidity of the soluble CMC becomes weak and it is difficult to diffuse into the pores to achieve the active sites of the enzyme at low temperature. However, at a high temperature, the immobilized cellulase shows higher stability. This should be because that a covalent bond between support and enzyme protects the conformation of cellulase during heating.

In order to further investigate the factors affecting the cellulase activity, the immobilized enzyme and free enzyme were used to hydrolyze 1% CMC solution for 50 minutes at 50 ºC with different pH (3, 4 and 5) and at pH=4 with different temperatures (50, 60 and 70 ºC). The concentration of glucose in the supernatant was measured every ten minutes. As the shows in Fig. 5-7, both the immobilized cellulase and free cellulase has the highest concentration of glucose at pH=4. The glucose produced by the free enzyme hydrolysis of CMC is higher than that of the immobilized enzyme. However, the change of pH has a greater impact on the process of free enzyme hydrolysis of CMC. And the inhibitory effect on enzyme activity is more obvious at pH=3. The immobilized enzyme shows a relatively stable state under these conditions. It should be attributed to the covalent bonds and CS layer which confirms the above analysis. Fig. 5-8 shows the effect of temperature on the hydrolysis process. For the free enzyme (Fig. 5-8b), the concentration of glucose shows a higher level at 60 and 70 °C during the initial hydrolysis. However, the enzyme may denature and its original structure could be destroyed at an excessively high temperature which inhibits enzyme activity. This is why the growth rate of the glucose concentration quickly tends to be flat at 70 °C. For

immobilized enzyme (Fig. 5-8a), due to the restriction of covalent bonds, as the temperature increases, its diffusion coefficient does not change much. Therefore, the immobilized enzyme exhibits similar glucose concentrations at different temperatures during the initial hydrolysis. Simultaneously, the binding of the covalent bond and the structure of the support could provide a stable environment in which the cellulase can maintain its structure during the hydrolysis process.

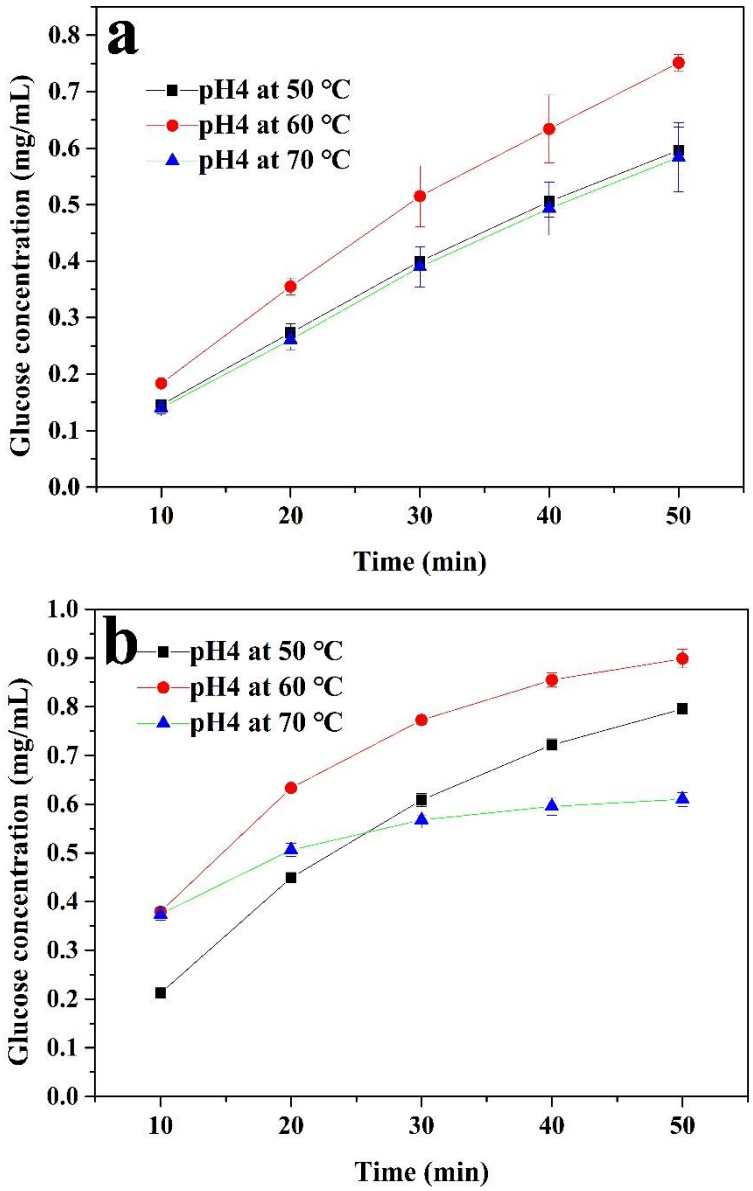

Fig. 5-8 The glucose produced from (a) immobilized cellulase and (b) free cellulase by hydrolyzing 1% CMC solution at pH=4 with different temperatures for 50 min.

5.3.3 Effect of CMC concentrations on cellulase activity

The Michaelis–Menten constant (K_m) and maximum reaction velocity (V_{max}) of enzyme are important kinetic parameters to know the tightness of the substrate and enzyme binding and the speed of the enzymatic reaction. The K_m and V_{max} were determined by Lineweaver-Burk plot and the results are shown in Table 5-2. The K_m value of the free and immobilized cellulase was 8.298 and 12.134 $g \cdot L^{-1}$, and the V_{max} value of them was 0.102 and 0.059 $g \cdot L^{-1} \cdot min^{-1}$, respectively. The increase in K_m and the decrease in V_{max} indicates that the binding tightness between immobilized enzyme and substrate has decreased compared with free enzyme. This is because of the steric hindrance and diffusion resistance generated by the support [40].

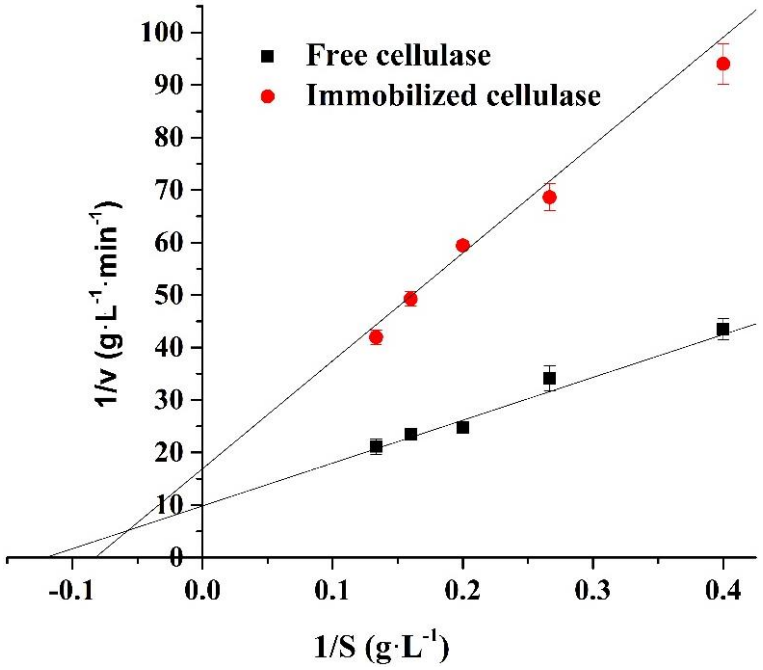

Fig. 5-9 Effect of CMC concentration on the cellulase activity (the cellulase concentration is 0.01 mg/mL, at pH4, 60°C for 5 min). Where the 1/V is the reciprocal of the enzymatic reaction rate and the 1/S is the reciprocal of substrate concentration.

5.3.4 Reusability of the immobilized cellulase

The reusability of the immobilized enzyme is one of the most key factors for

lowering the cost in the practical application. And it was evaluated by measuring the glucose yield in each hydrolysis cycle. The result shows in Fig. 5-10. As the number of uses increased, the glucose yield shows a slow decline and then becomes stable, whereas the initial glucose yield is 330.9 mg glucose/g CMC and keeps 86.0% after 10 repeated uses. The loss of glucose yield may cause by the protein denaturation or cellulase leakage during the hydrolysis process [5]. Moreover, it is possible because a large number of reaction products are deposited on the surface of the substrate and restricted the diffusion of proteins. In this part, the result suggests that the immobilized enzyme can be used several times with a high glucose yield which has potential in practical applications.

Table 5-2 Kinetic parameters of free and immobilized cellulase

	Km ($g \cdot L^{-1}$)	Vmax ($g \cdot L^{-1} \cdot min^{-1}$)
Free cellulase	8.298	0.102
Immobilized cellulase	12.134	0.059

In addition, the immobilized cellulase was stored in a refrigerator at 4°C for one month. Then the activity was tested. The result showed that the relative activity of the immobilized cellulase was 90.36% of the initial value. This indicates that the immobilized enzyme has good storage stability.

5.4 Conclusions

A chitosan/magnetic porous biochar support was successfully prepared by some simple methods. Cellulase was immobilized onto the support by covalent bonding using the GA agent. In the influence of pH and temperature tests, the relative activity trend of the immobilized enzyme was similar to that of the free enzyme. It seems that there are few alterations of the cellulase. And the optimum temperature and optimum pH of the immobilized enzyme and free enzyme were both 60 ° C and pH4. However, the immobilized cellulase showed high reusability: the remaining 86.0 % of initial glucose

productivity after 10 cycles. It has potential in practical applications based on the enzymatic performance of this immobilized cellulase, but it still needs more discussion.

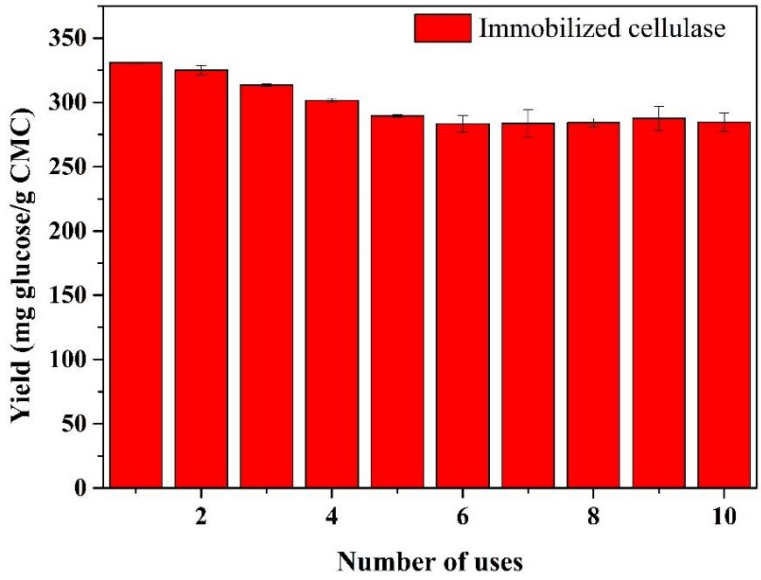

Fig. 5-10 Effect of recycle on the glucose productivity of immobilized cellulase. Each cycle is performed at pH 4 and 60 ºC for 24 h.

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Chapter 6 Conclusions

Porous biochar prepared in this work has a high specific surface area (~1600 m^2/g) and different types of pore structures. In addition, porous biochar has the advantages of abundant sources, simple preparation method, and low cost. Which could be emerging as a promising support to immobilize enzymes. However, the insufficient of reactive groups and hydrophilic groups, and inconvenient recovery limit its application in the immobilization of hydrophilic enzymes. To overcome these problems, chitosan modification was used to improve the biocompatibility of porous biochar and provided the required reactive functional groups. Magnetic modification can ensure that the immobilized cellulase can be recycled and reused conveniently and quickly.

In chapter 1, the research background and the construction of this thesis are described. The objectives of the research are to study the preparation of functional porous biochar and its application for cellulase.

In chapter 2, the properties of materials used in this thesis, experimental methods, and characterization are presented.

In chapter 3, three kinds of supports for cellulose immobilization were prepared by coating different amount of chitosan on the surface of porous biochar derived from sugarcane bagasse. The amount of chitosan has influence on morphology and particle size of supports as well as cellulase loading amount, activity recovery, thermal stability and reusability of immobilized cellulase. All the three kinds of immobilized cellulase showed a good reusability. Especially for C@CS25-cellulase, it retained the morphology of porous biochar well and had an activity recovery of 67%. In addition, it remained 90.8% glucose productivity after 10 cycles. According to the results, these supports can be recommended as ideal supports to immobilize enzyme.

In chapter 4, the calcination method was used for the preparation of magnetic polyporous biochar. Calcination temperature and time show direct impacts on the formation of C/γ-Fe_2O_3. As the temperature increases, the amount of crystallization increases. However, higher temperature or long-term calcination would destroy the

structure of γ-Fe_2O_3. The optimum condition is confirmed: calcination temperature is 500 °C and calcination time is 60 min. The large specific surface area (422.6 m^2g^{-1}) of 1# has a high zeta potential (-37.94±5.35 mV), which shows a good cellulase adsorption capacity (266 mg cellulase/g adsorbent) after 12 h of adsorption at 50 °C. Simultaneously, the immobilized cellulase retains 73.6% of the activity compared with free cellulase (at 50 °C and pH value = 4). Also, it has some reusability (maintaining 51.7% glucose yield after three uses).

In chapter 5, chitosan/magnetic porous biochar support was successfully prepared by some simple methods. Cellulase was immobilized onto the support by covalent bonding using the GA agent. In the influence of pH and temperature tests, the relative activity trend of the immobilized enzyme was similar to that of the free enzyme. It seems that there are few alterations of the cellulase. And the optimum temperature and optimum pH of the immobilized enzyme and free enzyme were both 60 ° C and pH4. However, the immobilized cellulase showed high reusability: the remaining 86.0 % of initial glucose productivity after 10 cycles. It has potential in practical applications based on the enzymatic performance of this immobilized cellulase, but it still needs more discussion.

Publications

1. 投稿論文

(1) Chao Yang, **Haodao Mo**, Limin Zang, et al. Surface functionalized natural inorganic nanorod for highly efficient cellulase immobilization[J]. *RSC Advances*, 2016, 6(80): 76855-76860. (IF=3.01)

(2) Baiyi Chen, Jianhui Qiu, **Haodao Mo**, et al. Synthesis of mesoporous silica with different pore sizes for cellulase immobilization: pure physical adsorption[J]. *New Journal of Chemistry*, 2017, 41(17): 9338-9345. (IF=3.24)

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2. 国際会議

(1) Baiyi Chen, **Haodao Mo**, Jianhui Qiu, Kazushi Ito, Eiichi Sakai, Nobuhiro Kanazawa. Candle soot as a template for transparent, durable and superhydrophobic surface and its application in oil-water separation. The 12th China-Japan Joint Conference on Composite Materials (CJJCC-12), Kochi, Japan, September 14-18,

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(2) **Haodao Mo**, Jianhui Qiu, Eiichi Sakai, Kazushi Ito, Chao Yang, Limin Zang. Magnetic Biochar for High Performances Immobilized Cellulase. The 13th China-Japan Joint Conference on Composite Materials (CJJCC-13), Lanzhou, China, October 20-22, 2018

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(4) **Haodao Mo**, Jianhui Qiu, Chao Yang, Limin Zang, Eiichi Sakai. Analysis of the Formation Factors of Polyporous C/γ-Fe_2O_3 and Its Application of Cellulase Adsorption. The 1stInternational Symposium on Advanced Materials Science and Engineering (AMSE-1), Akita, Japan, August 20-25, 2019

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3. 国内会議

(1) 莫 好道、楊 超、邱 建輝、境 英一、伊藤 一志、臧 利敏. グルタルアルデヒドによりセルラーゼを固定化したアタパルジャイト/キトサン複合材料の創製. 日本機械学会・東北支部第52期秋季講演会. 2016-9-17. 秋田市.

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