秋田県立大学大学院博士学位論文

Study on Preparation of Functionalized Magnetic Supports for the Cellulase Immobilization

（機能性磁性担体の創製およびセルラーゼの固定化に関する研究）

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Abstract

Bioethanol from lignocellulosic biomass is one of the most promising biofuels to lessen the environmental pollution generated by burning of fossil fuels. Conversion of lignocellulosic materials to bioethanol is performed by cellulase, which are biocatalysts with some excellent properties (high activity, selectivity and specificity) that can allow complex chemical processes to be carried out under the mild experimental and environmental conditions. In spite of its natural stability, the use of cellulase is limited by the enzyme inactivation during the hydrolysis process and the difficulty to separate cellulase from the solution, which limits the further industrial applications of enzymatic hydrolysis of lignocellulosic biomass. Immobilization of enzymes on magnetic supports has been increasingly used in industrial bioprocesses due to improved stability, the potential for modification of the catalytic properties, their recyclability, and in certain cases higher activity or selectivity. Therefore, development of biocompatible magnetic supports and effective immobilization strategies were carried out to ensure the complete and large-scale use the biocatalytic potential of the added cellulase.

In chapter 1, the research backgrounds, research significance, summary of the research and the construction of this thesis are described. The objectives of the research are to study synthesis of magnetic supports and strategy of immobilized enzyme.

In chapter 2, the properties of magnetic supports and immobilized enzyme are presented. The experimental methods and characteristics are also presented in this chapter.

In chapter 3, functionalized magnetic silica nanospheres were prepared in two steps: by silica coating magnetite nanoparticles and then making an amino-silane modification of the silica-coated magnetite particles. The effects of different amounts of tetraethylorthosilicate on the morphologies and magnetic properties of the silica-coated magnetite particles were investigated and the structures and properties of the functionalized magnetic silica nanospheres were characterized. Then functionalized magnetic silica nanospheres prepared using 5 mL tetraethylorthosilicate were used as supports for immobilization of cellulase. The results indicated that immobilized cellulase exhibited better resistance to high temperature and pH inactivation in comparison to free cellulase. The amount of immobilized cellulase was 92 mg/g support. Furthermore, the activity of the immobilized cellulase was still 85.5 % of the initial activity after 10 continuous uses, demonstrating the potential of this immobilized

cellulase for large-scale biofuel production.

In chapter 4, functionalized magnetic nanospheres were prepared by co-condensation of tetraethylorthosilicate with three different amino-silanes: 3-(2-aminoethylamino propyl)-triethoxysilane (AEAPTES), 3-(2-aminoethylamino propyl)-trimethoxysilane (AEAPTMES) and 3-aminopropyltriethoxysilane (APTES). Then three functionalized magnetic nanospheres were used as supports for immobilization of cellulase. The three functionalized magnetic nanospheres with core-shell morphologies exhibited higher capacity for cellulase immobilization than unfunctionalized magnetic nanospheres. The increasing of surface charge of functionalized magnetic nanospheres leads to an enhancement of the capacity of cellulase immobilization. Particularly, AEAPTMES with methoxy groups was favored to be hydrolyzed and grafted on unfunctionalized magnetic nanospheres than the others. AEAPTMES functionalized magnetic nanospheres with the highest zeta potential (29 mV) exhibited 87% activity recovery and the maximum amount of immobilized cellulase was 112 mg/g support at concentration of initial cellulase of 8 mg/mL. Immobilized cellulase on AEAPTMES functionalized magnetic nanospheres had higher temperature stability and broader pH stability than other immobilized cellulases and free cellulase. In particular, it can be used in about 40 °C, demonstrating the potential of biofuel production using this immobilized cellulase.

In chapter 5, the novel magnetic composite microspheres were prepared by copolymerization of glycidyl methacrylate and methacryloxyethyl trimethyl ammonium chloride using 1,1-diphenylethylene as radical control agent in the presence of Fe_3O_4 nanoparticles. The structures and magnetic properties of magnetic composite microspheres were characterized by scanning electron microscopy, Fourier transform infrared spectroscopy and thermogravimetric analysis. The results indicate that the polymer chains had been effectively grafted onto the surface of Fe_3O_4 nanoparticles. Cellulase was immobilized on magnetic composite microspheres by electrostatic adsorption and covalent binding with 153 mg/g supports at concentration of initial cellulase of 6 mg/mL. The relative activity of the immobilized cellulase was still 72% of the initial activity after 10 continuous uses.

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

Content

Chapter 1 Introduction

1.1. Background

Lignocellulosic waste materials obtained from energy crops, wood and agricultural residues, represent the most abundant global source of renewable biomass [1, 2]. In recent years, turning from burning of lignocellulosic waste materials to production of ethanol from lignocellulosic waste materials seems to be a promising approach to gain energy from waste biomass and partly reduce dependence on fossil fuels while contributing to the greenhouse gas effect and improving urban air quality [3, 4]. Bioethanol has gained much attention especially and its global production showed an upward trend over the last 25 years. Bioethanol production capacity in 2005 and 2006 were about 45 and 49 billion liters per year in the world, respectively and total output in 2015 is forecast to reach over 115 billion liters [5].

Utilization of lignocellulosic material for the fermentative biofuel production, cellulose has to be degraded into fermentable sugars using enzymatic methods due to economic and environmental factors [6]. Cellulase is an enzymatic complex composed of 1,4-β-D-glucanases or endoglucanases, exo-1,4-β-D-glucanases or cellobiohydrolases and 1,4-β-D-glucosidases [7]. These enzymes allows for efficient conversion of renewable natural resources into fermentable sugars. However, the largest challenges in optimizing cellulosic ethanol production and hence increasing the feasibility of a large-scale industrial application are enzyme stability and reusability, as well as the high cost associated with the initial purification of the enzyme mix from natural sources [8]. One approach to increase the performance of the enzymatic process is to immobilize the enzymes on supports which renders them reusable and hence reduces the enzyme-related operating costs.

1.2. Immobilization of enzyme

Immobilized enzymes have been used in a variety of scientific and industrial applications [9]. The economic importance of immobilization has resulted in considerable research for industrial applications. In addition, immobilization of enzymes provide other advantages including improved stability, the potential for modification of the catalytic properties, the prevention of microbial growth, and in certain cases higher activity or

selectivity [10].

1.2.1. Principle and method of immobilized enzyme

Immobilization of enzyme on supports has been achieved using a diverse range of methodologies [11-13]. Immobilization methods have been classified on the basis of the interaction between enzyme and the support used for immobilization. They are mainly based on chemical and physical mechanisms. Their basic aspects are summarized. Physical methods involve: (i) entrapment, (ii) adsorption. Chemical immobilization methods mainly include: (i) cross-linking, (ii) covalent bonds. Fig. 1-1 [11] illustrates the basic methods of immobilization.

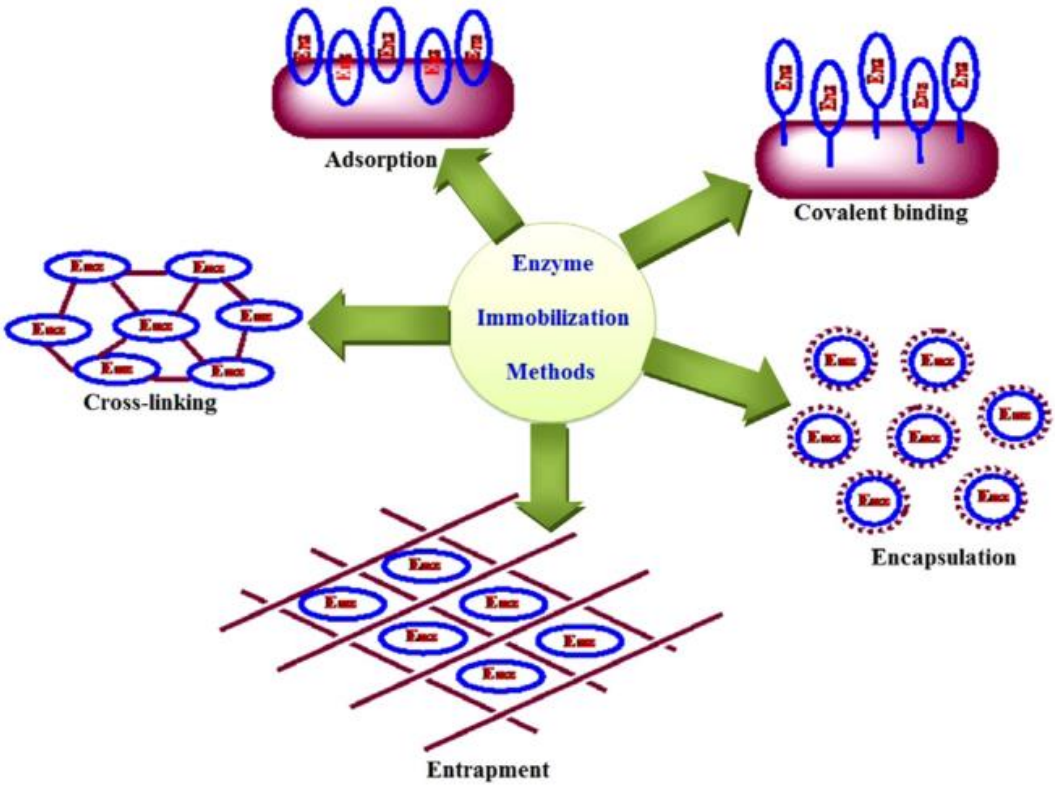

Fig. 1-1. Basic enzyme immobilization methods [11]

(a). Entrapment

Enzyme immobilization by entrapment has the benefit of a wide applicability and may provide relatively small perturbation of the enzyme native structure and function.

Entrapment of an enzyme entails capture of the enzyme within the inner cavities of a matrix or capsule of sol-gel. Alginate, chitosan, polyacrylamide gel and silica gel are often used as supports for enzyme immobilization. β-galactosidase was encapsulated in alginate-gelatin-calcium phosphate hybrid capsule that effectively prevented the leakage of β-galactosidase. β-galactosidase immobilized in hybrid capsules exhibited the broadest temperature and pH ranges in comparison to free enzyme [14]. Lipase can be immobilized successfully by entrapment in chitosan gel beads. The obtained beads were about 2 mm diameter, and the enzyme entrapment efficiency was 44–48%. Lipase activity in chitosan beads was higher compared to lipase entrapped in alginate beads [15]. Enzyme was entrapped within a polyacrylamide gel, obtained by polymerization/cross-linking of acrylamide in the presence of the enzyme [16]. Lipase was successfully entrapped into various cellulose–biopolymer composite hydrogels by using a biocompatible ionic liquid, 1-ethyl-3-methylimidazolium acetate ([Emim][Ac]). Cellulose–biopolymer composite hydrogel beads were prepared by cellulose mixed with co-dissolution of biopolymers in [Emim][Ac] and adding lipase in biopolymer solution to form of biopolymer hydrogel. Immobilization yields of cellulose, cellulose–carrageenan, cellulose–chitosan, cellulose–agarose, and cellulose–agar bead were 35.0, 9.6, 39.7, 41.4, and 52.6%, respectively. Cellulose–biopolymer composite hydrogels were good supports for entrapment of enzymes and have many potential applications due to their inherent excellent biocompatibility and biodegradability [17].

Entrapment by mesoporous silica is attributed to its high surface area, uniform pore distribution, tunable pore size and high adsorption capacity. The cellulase was encapsulated onto mesoporous materials (SBA-15) of various pore size (5.4-11 nm). Encapsulated cellulase on mesoporous materials with a pore size of 8.9 nm showed the highest enzyme activity and was more accessible to the substrate than others. Encapsulated cellulase on mesoporous materials exhibited the enhanced the stability over free cellulase [18]. Wang et al. described an effective method to encapsulate enzymes by using mesoporous silica for enzyme immobilization. Encapsulation resulted in enhanced enzyme properties. Catalase also exhibited enhanced stability in reaction conditions over a wide pH rang (pH 5-10) and retained an activity of 70% after 25 successive batch reactions [19]. The entrapment method for immobilizing enzyme is fast, cheap, and usually involves mild conditions. But the biggest disadvantage of entrapment is the mass transfer limitation, so the lipase is only effective for low molecular weight substrates [20, 21].

(b). Adsorption

Physical adsorption of cellulase on solid supports is perhaps the most straightforward technique to achieve immobilization [22]. Lower costs and relatively non-toxic mode of attachment are some of the major advantages of using this technique. Vander–Waal forces of attraction, hydrogen bonding, hydrophobic and electrostatic attraction are some of the common modes of attachment of the protein molecules on the supports [23].

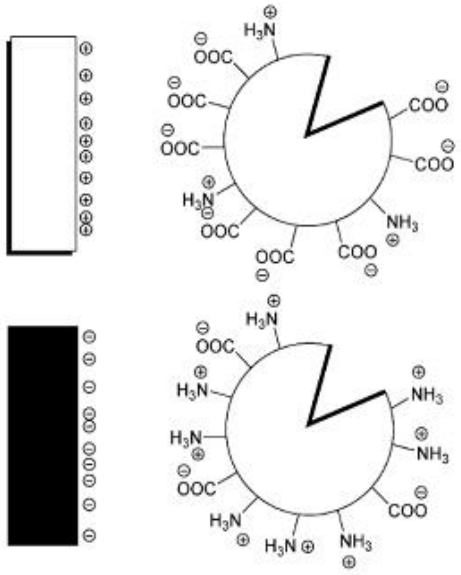

Fig. 1-2. Immobilization of enzyme by adsorption [23]

Electrostatic groups on the surface of a support may lead to attraction of individual ionic amino acids on the enzyme surface [24]. This method is based on alternate supports and enzyme with opposite charges as shown in Fig. 1-2 [23]. Carbon nanotube (CNT) with its high surface area can serve as a very good support for immobilization of enzyme. β-glucosidase (GOD) has positively charged protein segments which can be electrostatically assembled on negatively charged carbon nanotubes. β-glucosidase can be immobilized successfully by electrostatic adsorption on carbon nanotube [25]. In addition, CNT is modified with a charged layer, such as poly(allylamine), poly(L-lysine), poly(ethyleneimine), poly(dimethyldiallyl ammonium chloride) (PDDA), poly(allylamine hydrochloride) and chitosan (or chitosan derivatives). The most commonly used polyanions are poly(styrenesulfonate) (PSS), poly(vinylsulfonate),

poly(anilinepropanesulfonic acid), poly(acrylic acid) and poly(methacrylic acid). This negatively (or positively) charged surface is then immersed in a polycation (or polyanion) solution to form the first positively (or negatively)-charged layer. Negatively-charged enzymes are immobilized on the polycationic electrode through electrostatic forces. For example, negatively GOD molecules were alternatively adsorbed onto the layer PSS/PDDA/CNTs until the desired number of layers was obtained [26]. The adsorption of GOD onto the layer PSS/PDDA/CNTs can keep the bioactivity of enzyme and prevent enzyme leakage.

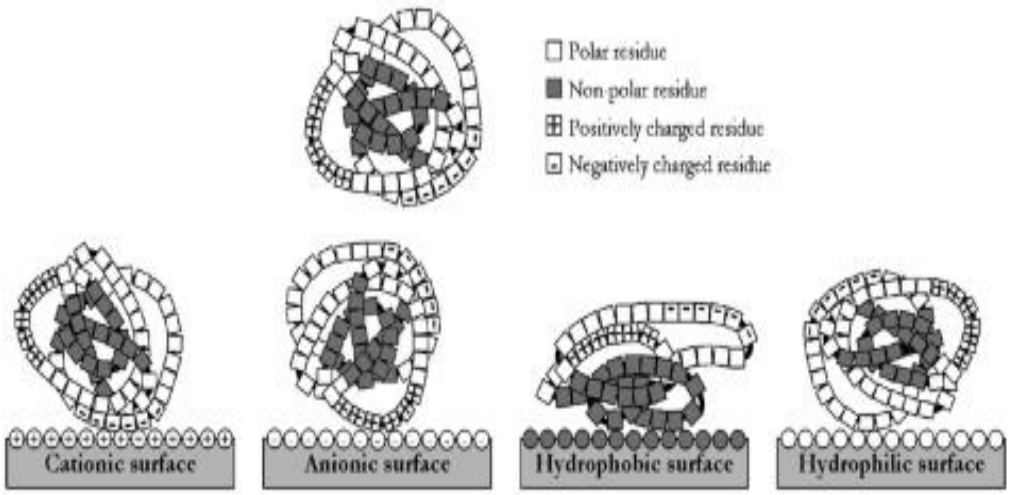

Fig. 1-3. Mechanisms of protein adhesion [30]

Surface modification using organic functional groups has been found to be useful for the immobilization and adsorption of enzymes to the surface of the silica material [27, 28]. Mesoporous silica materials (FDU-12) modified using the type of organosilane were used to immobilize cellulase [29]. Phenyl (PTMS)- and thiol (MPTMS) functionalized FDU-12 mesoporous silicas had a very low adsorption capacity of proteins because of their small pore sizes. Amino (APTES)-functionalized FDU-12 mesoporous silica showed the highest adsorption amount of proteins, which was due to them carrying a positive charge. Electrostatic attraction occurs between enzyme and amino-functionalized FDU-12 mesoporous silica. Cellulase immobilization on vinyl (VTMS) functionalized FDU-12 mesoporous silica appeared to be the most promising approach, since it occurred with high efficiency, maintained enzyme activity, and provided temporal enzyme stability. The reason for this phenomenon is probably the

hydrophobic interaction between certain (hydrophobic) protein domains and vinyl moieties. The mechanisms of protein adhesion by hydrophobic and electrostatic attraction were shown in Fig. 1-3 [30]. Although this immobilization method causes little or no enzyme inactivation, this technique presents drawbacks: enzymes are loosely bound to the support and desorption of the enzyme resulting from changes in temperature, pH and ionic strength, appears to be the main problem. Thus, adsorbed enzymes suffer from poor operational and storage stability. Another drawback is the non-specific adsorption of other proteins or substances.

(c). Cross-linking

Cross-linking method refers to a three-dimensional network structure through the interaction within enzyme, coupling reagent, and carrier. This method is attractive due to its simplicity and the strong chemical binding achieved between biomolecules. Immobilization of enzymes by cross-linking with glutaraldehyde, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide is well-known approach. A gravimetric glucose biosensor was also reported for selective detection of blood glucose levels. GOD was immobilized onto cantilever surface by cross-linking with glutaraldehyde and BSA [31]. An amperometric glucose biosensor was developed by immobilizing GOD onto ZnO nanotube-modified electrode by crosslinking [32]. Magnetic cross-linked enzyme aggregates of alpha amylase were prepared by chemical cross-linking of enzyme aggregates with amino functionalized magnetite nanoparticles [33]. Scanning electron microscopy analysis showed that CLEAs and magnetic CLEAs were spherical structures. The magnetic CLEAs also enhanced the thermal stability and storage stability. Moreover, the magnetic CLEAs retained 100% initial activity even after 6 cycles of reuse.

Enzymes in solution can be crosslinked to form carrier-free CLEA. Fig. 1-4 shows that aggregation and crosslinking of an enzyme to prepare a CLEA [23]. Cellulase was crosslinked by glutaraldehyde to obtain a heat-stable enzyme preparation for rice hull cellulose hydrolysis [34]. CLEA having 15% more activity than free enzyme was obtained which also had considerable improvement in heat stability at 65°C and 70°C. Whereas the free enzyme lost 80% of its activity in 4 h at 65°C, CLEA lost only 30% activity.

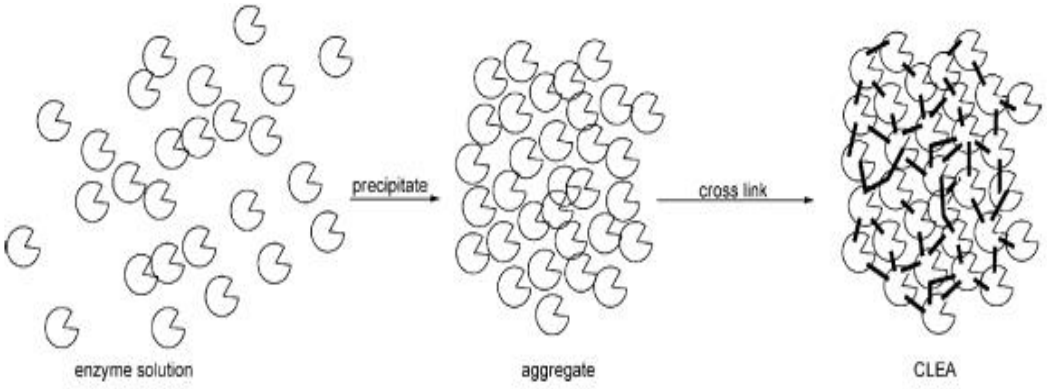

Fig. 1-4. Aggregation and crosslinking of an enzyme to prepare a CLEA [23]

(d). Covalent binding

Covalent binding of enzymes to supports is a popular chemical immobilization method based due to strong stable enzyme attachment, reduction enzyme deactivation rates and usefully alterrtion enzyme specificity [35]. Covalent of enzyme to a carrier biocatalysts are bound to the surface through functional groups that they contain and that are not essential for their catalytic activity. The binding of the enzymes to the solid support is generally carried out by initial activation of the surface using multifunctional reagents (e.g. glutaraldehyde or carbodiimide) [36], followed by enzyme coupling to the activated support, then the removal of excess and unbound biomolecules.

Numerous protocols for activating solid surfaces have been described. Carbodiimides (EDC) allow the binding between the carboxyl or hydroxy groups of support and the amino function of an enzyme. In addition, carbodiimides allow the binding between the amino group of support and the carboxyl function of an enzyme (Fig. 1-5). Enzymes can also be covalently immobilized onto the ends of single walled carbon nanotubes (SWCNTs) by using EDC to promote amide linkages between carboxyl- terminated nanotubes and lysine residues of the enzyme for H_2O_2 detection [37]. Immobilization of lipase to chitosan beads by activating the hydroxyl groups of chitosan using EDC coupling agent has been successfully developed and the process appreciably increased the activity of lipase immobilized to wet chitosan beads [38]. An actual hydrolytic activity (Vmax) of 117.23 U/g-chitosan and relative specific activity of 2.110%. The stability (pH, thermal, storage and reuse) of the immobilized lipase preparations was also enhanced. Increased activity and operational stability of lipase immobilized on a

cheap support like chitosan by the activation of hydroxyl groups makes it ideally suitable for the economic production of immobilized biocatalysts for the industrial applications like the manufacture of fatty acid by the hydrolysis of triglyceride.

The binding of cellulase enzyme complex to magnetic (Fe_3O_4) nanoparticles via carbodiimide activation was investigated [39]. The maximum weight ratio (mg bound enzyme/mg nanoparticles) achieved to 0.16 was determined and the enzyme to support saturation point occurred at a weight ratio of 0.02 as it attained a maximum activity value of 62.7 IU/mg. Thermal measurements for the nanoparticles indicated increased stability over a broader range of temperatures, and exhibited the maximal activity at 50 °C. The ionic forces between the enzyme and support surface caused a shift in the optimum pH from 4.0 to 5.0. The reusability of the immobilized cellulase complex was 30.2% of the corresponding free enzyme activity after six recycles. With the covalent bond method, the strong interactions between the lipase and the support, which is due to the fact that the formation of multiple covalent bonds between the enzyme and the support reduces conformational flexibility and thermal vibrations thus preventing protein unfolding and denaturation.

Glutaraldehyde are employed for covalent immobilization of enzyme by activation method (Fig. 1-6). This method is attractive due to its simplicity and the strong chemical binding achieved between biomolecules. Cellulase was immobilized on this magnetic support using glutaraldehyde as a coupling agent [40]. The immobilized cellulase has high magnetic sensitivity of (46.6 emu/g) that makes the separation of cellulase from reaction mixture easy. The amount of cellulase on Fe−chitosan nanoparticles with an activity of 5.23 IU/mg cellulase was 112.3 mg/g. The thermal stability of the immobilized cellulase was preferable to the free cellulase. The immobilized cellulase retained 50% of its initial activity after 10 cycles. Meanwhile, the glucose productivity during 24 h remained higher than 80% of the original after 10 cycles. Lipase was immobilizated on nano-sized magnetic support using glutaraldehyde as a coupling agent [41]. Experimental data showed that the immobilized lipase exhibited good thermal stability and reusability. The lipase loading amount and activity recovery were found to be 43.6 mg/g support and 58.2%.

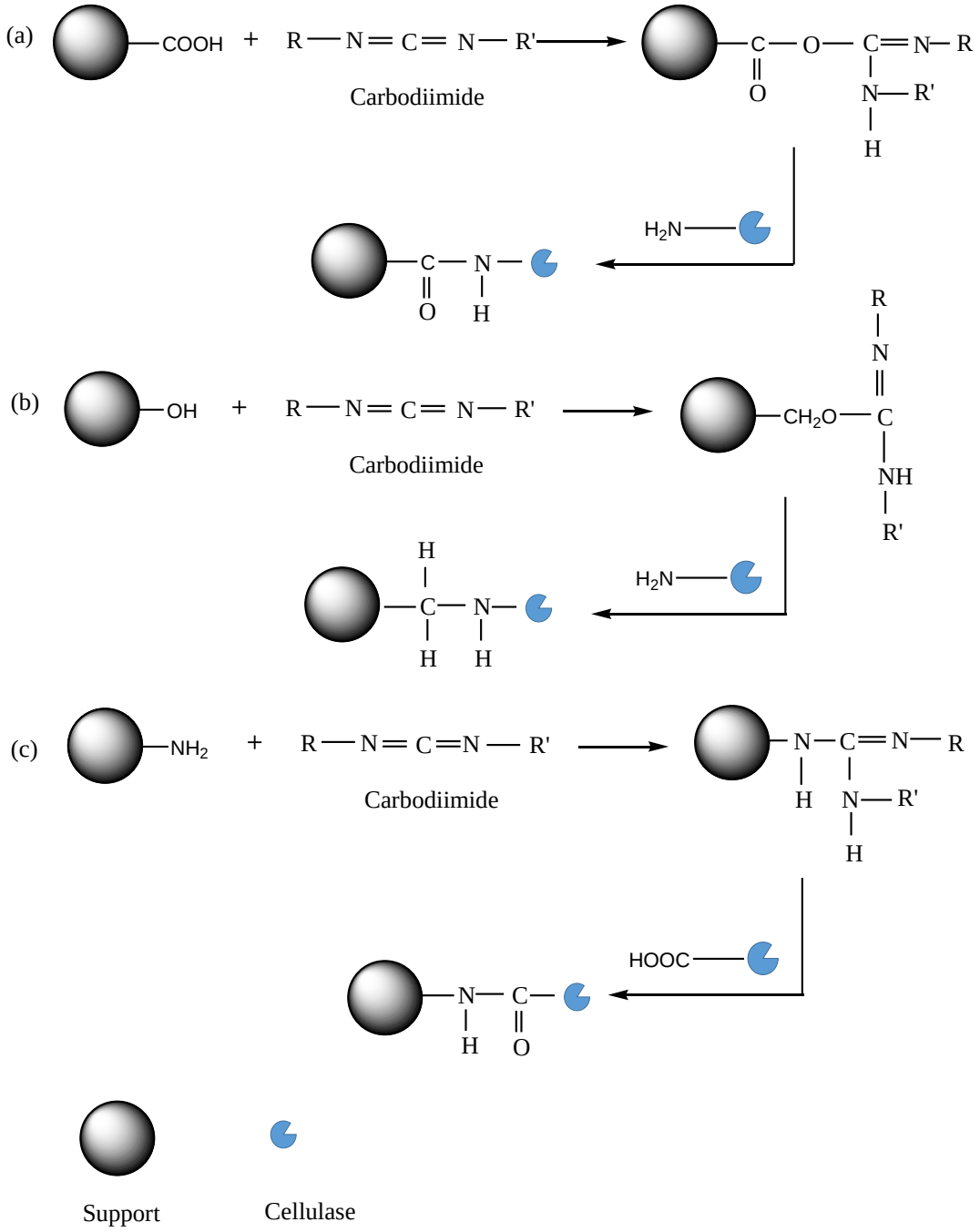

Fig. 1-5. Enzyme immobilized on supports with diverse functional group by carbodiimide (a) carboxyl, (b) hydroxyl, (c) amino groups

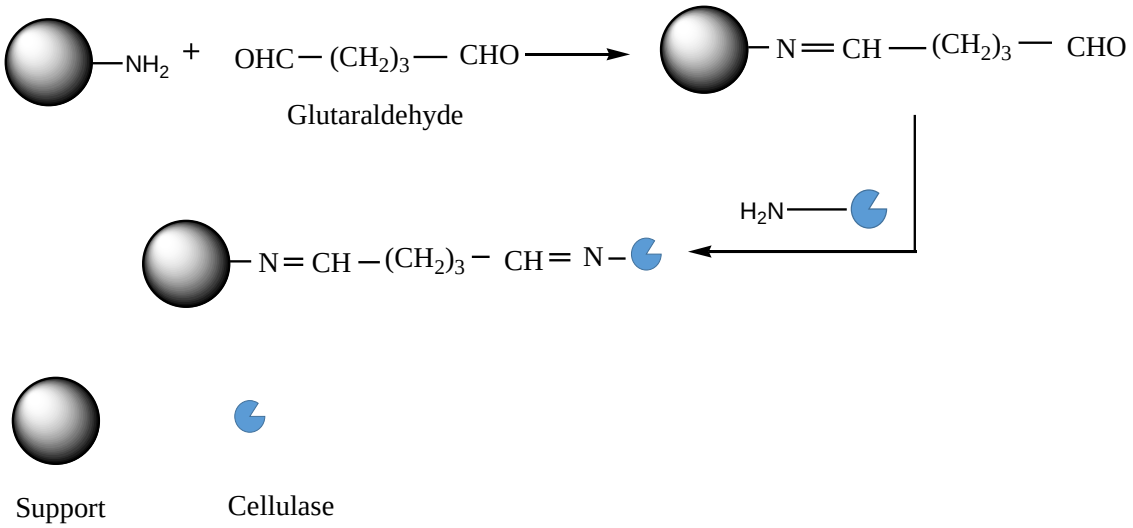

Fig. 1-6. Enzyme immobilization by glutaraldehyde coupling

1.3. Magnetic particles

Magnetic particles are the most often used supports because of their good biocompatibility, magnetic susceptibility, low toxicity, and ease of preparation in the desired size [42, 43]. Magnetic particles are important material for a wide range of application, such as bioseparation, biocatalysis, virus detection and biomolecules immobilization, which was due to the ease of separating biomolecules from reaction solutions, their recyclability and preventing of biomolecules dissociation [44, 45].

1.3.1. Nanoparticle design considerations

Magnetic particles design requires fundamental understandings of the nature of the nanostructure as (i) a biocompatible entity (morphology, hydrodynamic size, charge, and other surface properties) (ii) not harm the enzyme, and (iii) improvement of stability enzyme [46]. Here, we will consider the first of these areas, specifically looking at synthetic strategies of magnetic support to gain biocompatible magnetic support.

(a). Hydrodynamic size

Magnetic particles biodistribution appears to be significantly influenced by its physicochemical properties [47, 48]. Their importance relates to the fact that the characteristics of nanoparticles are different from those of bulk materials of the same composition, which is mainly because of size effects and the magnetic properties. The

immobilization of enzymes onto nano-scale materials provides a reduction in the size of the enzyme-support materials which generally improves the efficiency of immobilized enzymes; for example, regarding the surface area for the attachment of enzymes, higher enzyme loading per unit mass of particles [49].

(b). Shape

Magnetic particles are very promising candidates due to their biocompatibility and relatively easy to be coated with organic materials (e.g., polymers) or inorganic metallic (e.g., gold) to form core-shell structure, achieve good dispersion and make them biocompatible.

Coating magneticparticles with silica shell is a promising and important approach to immobilize enzyme. Silica coating of magnetic nanoparticles favors the dispersion of magnetic nanoparticles in liquid media and protects them from leaching in an acidic environment [50-52]. But, more importantly, the silica layer provides a chemically inert surface for applications. For example, cellulase could be immobilized irreversibly onto Si wafers due to hydrogen bonding between silanol and cellulase polar groups [53-56]. Cellulase was immobilized by sol-gel encapsulation, using magnetic sol-gel silica. Furthermore, coating magnetic particles with silica shell are suitable for further the attachment of various bioactive molecules on functionalized support by surface functionalization.

(c). Surface properties

Magnetic particles sol-gel, charge, and hydrophobicity can affect magnetic particles biodistribution by limiting or enhancing interactions of magnetic particles with the adaptive immune system [57]. Positively charged magnetic particles can also bind with non-targeted cells (typically negatively charged) leading to nonspecific internalization. Surface modification with molecules like the hydrophilic polyethylene glycol (PEG) have been shown to reduce the potential for opsonization through steric repulsion, prolonging NP circulation times [58]. Specifically, the utility of organic coatings will be properly addressed in later sections. Another important group of magnetic solids are synthesized from organic polymers. Commercial magnetic polymers including cellulose, polystyrene and polyacrylic derivatives have also been functionalized with groups or recognition compounds [59].

1.3.2. Preparation of magnetic carriers

Magnetic nanoparticles (Fe_3O_4) in themselves have some disadvantage: (a) magnetic nanoparticles have high chemical activity and get easily oxidized when exposed to air, (b) they have a large surface area and therefore possess high surface energies; as a result, they tend to aggregate so as to minimize the surface energies, (c) poor biocompatibility. To address such issues, the development of a proper surface coating to protect and keep the stability of magnetic nanoparticles (MNPs) is required. The strategies comprise grafting of or coating with organic molecules, including small organic molecules or surfactants, polymers, and biomolecules, or coating with an inorganic layer, such as silica, metal or non-metal elementary substance, metal oxide or metal sulphide [60]. Practically, it is worthy that in many cases the protecting shells not only stabilize the MNPs, but can also be used for further functionalization and binding of biomolecule. In order to take advantage of the MNP bioresponses and targeting techniques detailed above, to control the physicochemical properties of MNPs, we need to implement controlled synthesis and coating processes. In the following sections we will discuss some of the coating techniques used in the MNP field and detail the significant design parameters that can assist in synthesizing biocompatible MNPs.

(a). Silica coating

Silica is often used as coating material over the surface of MNPs as silica is chemically inert, promotes the dispersion of the MNPs, have a high surface silanol concentration which facilitates a wide variety of surface reactions and the binding of biomolecules (antigen-antibodies, peptides, proteins, nucleic acids, enzymes), metals and polymers [61]. The physicochemical mechanism of the silane agent modifying on the surface of MNPs according to Arkles [62] is depicted in Fig. 1-7.

The hydroxyl groups on the MNPs surface reacted with the alkoxy groups of the silane molecules leading to the formation of Si–O bonds and forming the terminal functional groups available for further immobilization of other biomolecules. In general, the behaviour of silica coated magnetic nanoparticles is related to the thickness of their respective silica shells. Normally, a thicker silica shell reduces the inter-particle interaction and superparamagnetism is preserved although it is also accompanied by a sacrifice in saturation magnetization. The reduction value could be attributed to the lower density of the magnetic component in the silanized nanoparticle sample [63]. For instance, after silica coating, saturation magnetization to decrease from 81.2 $emug^{-1}$ to

49.7 $emug^{-1}$ [64]. The 3-aminopropyltriethoxysilane (APTEOS), p-aminophenyltrimethoxysilane (APTES) and mercaptopropyltriethoxysilane (MPTES) agents are used for providing the amino and sulphydryl groups, respectively. Alkoxysilanes is a very useful approach to start MNPs functionalization. However, a drawback is the presence of some remaining silanol groups in the product owing to the incompletion of the dehydration reaction. These silanol groups are highly reactive [65, 66] and, unless they are endcapped with a suitable reagent, their presence might cause further condensation reactions during the period of storage and usage of the formed nanocomposites [65].

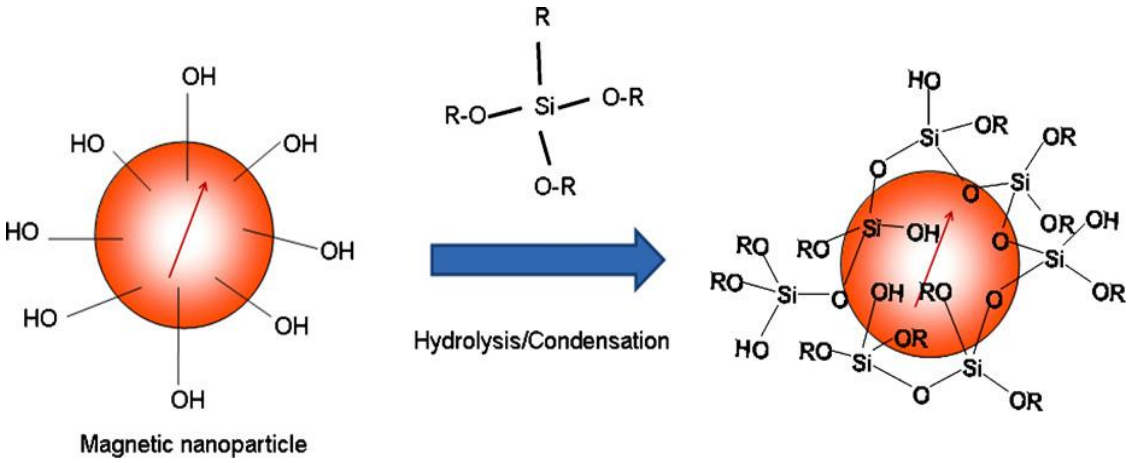

Fig. 1-7. Silane surface coating of a magnetic nanoparticle [62]

Chen and co-workers [67] have developed Fe_3O_4 magnetic nanoparticles coated estrone-imprinted polymer using a semi-covalent imprinting strategy. Synthesized Fe_3O_4 nanoparticles were firstly coated with a shell of silica using tetraethoxysilane (TEOS), after which the silica modified magnetic nanoparticles were reacted with a silane derivative of estrone to create a second shell of molecularly imprinted sol–gel. Estrone was extracted by hydrolysis. The resulting Fe_3O_4 magnetic nanoparticles coated estrone-imprinted polymer exhibit a much higher specific recognition and saturation magnetization. These hybrid nanoparticles have the potentialities for biochemical separations.

(b). PEG coating

PEG is a biocompatible linear synthetic polyether that can be prepared with a wide range of sizes and terminal functional groups [68]. They are neutral, hydrophilic

molecules in biological fluids, which helps to improve the dispersity of the MNPs they are bound to [69].

(c). Chitin and chitosan coating

Chitin is one of the world's most abundant, renewable organic resources. A major constituent of the shells of crustaceans, the exoskeletons of insects and the cell walls of fungi where it provides strength and stability. Chemically, chitin is composed of (1→4) linked 2-acetamido-2-deoxy-β-D-glucose units (or N-acetyl-d-glucosamine) [70], forming a long chain linear polymer (Fig. 1-8).

Chitosan, which is a poly-N-acetylglucosamine, is a transformed oligosaccharide obtained by the deacetylation of chitin, and is the second after natural cellulosic carbohydrate polymers in abundance. Since highly biocompatible and easily biodegradable, chitosan has been used as a raw material for many applications.

Magnetic chitosan microspheres were prepared with reversed-phase suspension methodology. Glutaraldehyde was used as cross-linking reagent for the enzyme immobilization [71]. The microspheres had spherical shape with smooth surface, and its particle size was 5.0 μm. Enzyme was immobilized on magnetic chitosan microspheres by adsorption and cross-linking with glutaraldehyde. The immobilized enzyme exhibited the maximal enzyme activity at pH 3.0. The optimal temperature for immobilized enzyme was 10 °C and 55 °C. The thermal, operational, and storage stabilities of the enzyme were enhanced after immobilization

Feng and co-workers [72] have developed magnetic chitosan prepared in a well spherical shape by the suspension crosslinking technique for cellulase immobilization. The optimum concentration of the cross linker and cellulase solution for the immobilization was 4% (v/v) and 6 mg/mL, respectively. The immobilized enzyme had a broader pH range, and higher temperatures stability than that of the free cellulase. Storage stability was enhanced after immobilization. The residual activity of the immobilized enzyme was 78% of original after 10 batch hydrolytic cycles, and the morphology of carrier was not changed.

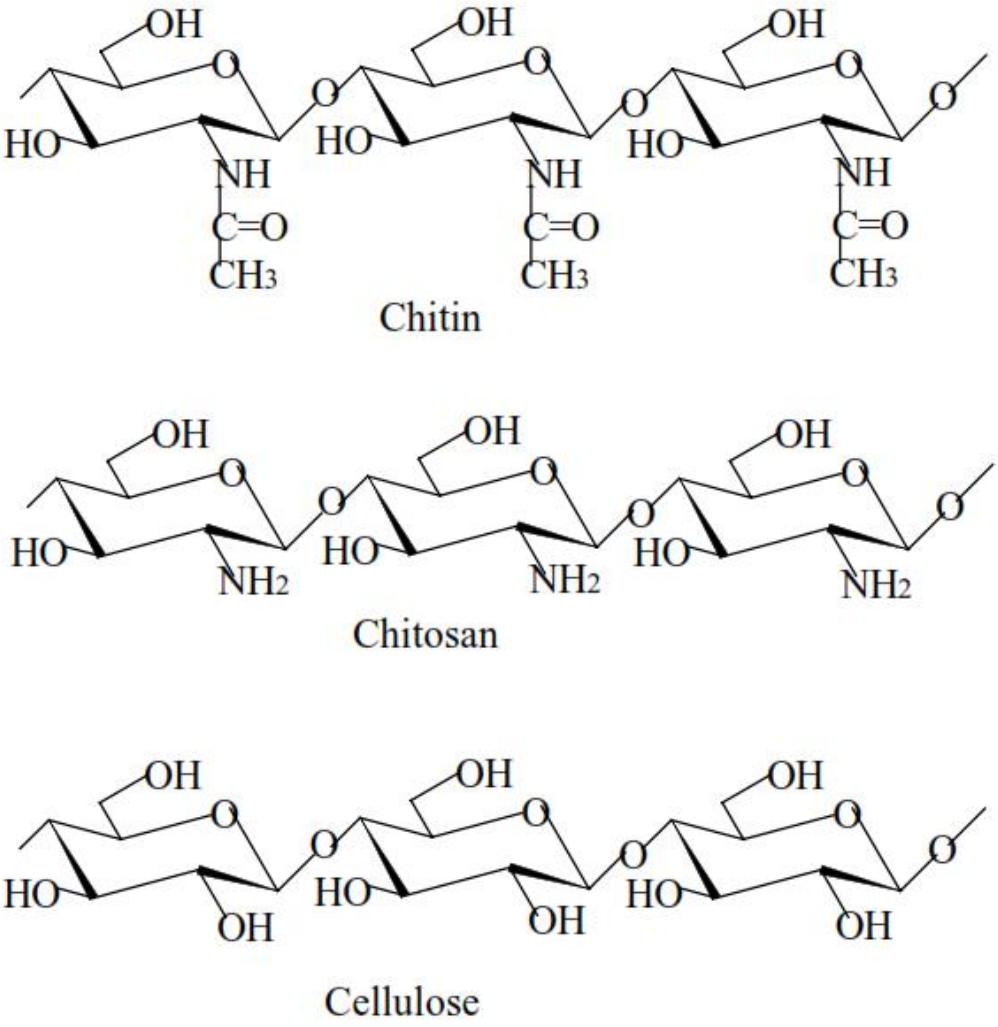

Fig. 1-8. Structure of chitin, chitosan and cellulose [70]

(d). Copolymers coating

Copolymers used as coating material over MNPs have attracted considerable interest because of their functionalities. The polymer chains offer flexibility and diversity to control the chemical composition and functional groups on the surface of nanoparticles. p(GMA) has been the most commonly employed polymeric material for grafting and modification due to excellent performance of epoxy group. The epoxy group are very stable at neutral pH values even under wet conditions and able to react with different nucleophilic groups of the protein (e.g. amino, hydroxyl, or thiolmoieties) to form extremely strong linkages with protein. For example poly(glycidyl methacrylate) (p(GMA)) grafted magnetic beads and p(GMA) grafted amine modified

chloromethylated resin, have been shown to have high immobilization capacities.

Micron-sized poly(styrene-co-glycidyl methacrylate) (PSt–GMA) fluorescent were synthesized via dispersion polymerization of styrene and glycidyl methacrylate [73]. The size of (PSt–GMA) fluorescent was 5.1 μm. Sufficient specific area was found to be 1.24 m^2/g microspheres. (PSt–GMA) fluorescent can be used as support for covalent immobilization of enzymes because of surface functional groups. The enzyme immobilization amount was approximately 11 mg/g microspheres. The immobilized enzymes retained approximately 28 to 34% activity, as compared with free enzymes, without pronounced alteration of the optimum pH and temperature.

Magnetic bead was prepared from the monomers glycidylmethacrylate (GMA) and methylmethacrylate (MMA) via suspension copolymerization in the presence of ferric ions [74]. The beads were sieved and 100–150 μm size of fraction was used in enzyme immobilization. The specific surface area of the magnetic beads was measured by the BET method and was found to be 16.2 m^2/g beads. The resulting magnetic beads were used for the covalent immobilization of lipase via glutaraldehyde activation and glutaraldehyde was also acted a 5-carbon spacer arm. The maximum lipase immobilization on magnetic poly(GMA–MMA) was 23.4 mg g^{-1}. The activity yield of the lipase immobilized on the spacer-arm attached magnetic beads was up to 81%.

N. Milosavić [75] succeeded in the immobilization of 190 mg of periodate oxidized glucoamylase per gram of poly (GMA-co-EGDMA). The covalently immobilized enzyme had a specific activity of 1100 U/g. The temperature and pH optimum as well as kinetic parameters were determined. During continuous use in a packed bed reactor over a period of 4 weeks the immobilized enzyme produced 1300 kg of glucose per 1 L of reactor volume without any decrease in its activity.

1.4. Purpose of this research

The use of cellulase is limited by the enzyme inactivation during the hydrolysis process and the difficulty to separate cellulase from the solution, which limits the further industrial applications of enzymatic hydrolysis of lignocellulosic biomass. In addition, there are only a few reports about magnetic particles being used as supports for immobilization of cellulase to produce bioethanol. In this study, magnetic supports for enzyme immobilization were developed and effective immobilization strategies were studied to ensure the complete and large-scale use the biocatalytic potential of the added

cellulase.

In chapter 1, the research backgrounds, research significance, summary of the research and the construction of this thesis are described. The objectives of the research are to study synthesis magnetic supports and strategy of immobilized enzyme.

In chapter 2, the experimental material are presented. The experimental methods and characteristics are also presented in this chapter.

In chapter 3, functionalized magnetic silica nanospheres were prepared in two steps: by silica coating magnetite nanoparticles and then making an amino-silane modification of the silica-coated magnetite particles. The effects of different amounts of tetraethylorthosilicate on the morphologies and magnetic properties of the silica-coated magnetite particles were investigated and the structures and properties of the functionalized magnetic silica nanospheres were characterized. Then functionalized magnetic silica nanospheres prepared using 5 mL tetraethylorthosilicate were used as supports for immobilization of cellulase. Then, the properties of immobilized cellulase were studied.

In chapter 4, functionalized magnetic nanospheres were prepared by co-condensation of tetraethylorthosilicate with three different amino-silanes: 3-(2-aminoethylamino propyl)-triethoxysilane (AEAPTES), 3-(2-aminoethylamino propyl)-trimethoxysilane (AEAPTMES) and 3-aminopropyltriethoxysilane (APTES). Then three functionalized magnetic nanospheres were used as supports for immobilization of cellulase. The properties of immobilized cellulase were studied.

In chapter 5, the novel magnetic composite microspheres were prepared by copolymerization of glycidyl methacrylate and methacryloxyethyl trimethyl ammonium chloride using 1,1-diphenylethylene as radical control agent in the presence of Fe_3O_4 nanoparticles. The structures and magnetic properties of magnetic composite microspheres were characterized. Then magnetic composite microspheres were used as supports for immobilization of cellulase. The properties of immobilized cellulase were studied.

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

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Chapter 2 Materials, Experiment and Characterizations

2.1. Materials

2.1.1. Cellulase

Meiji Acremonium cellulase (EN, 15-20 nm) was purchased from Meiji Seika Pharma Co., Ltd., Tokyo, Japan. The average diameter of cellulase is approximate 15-20 nm (Fig 2-1). Hydrolysis of lignocellulosic materials using cellulases represents the most effective method to liberate simple sugars.

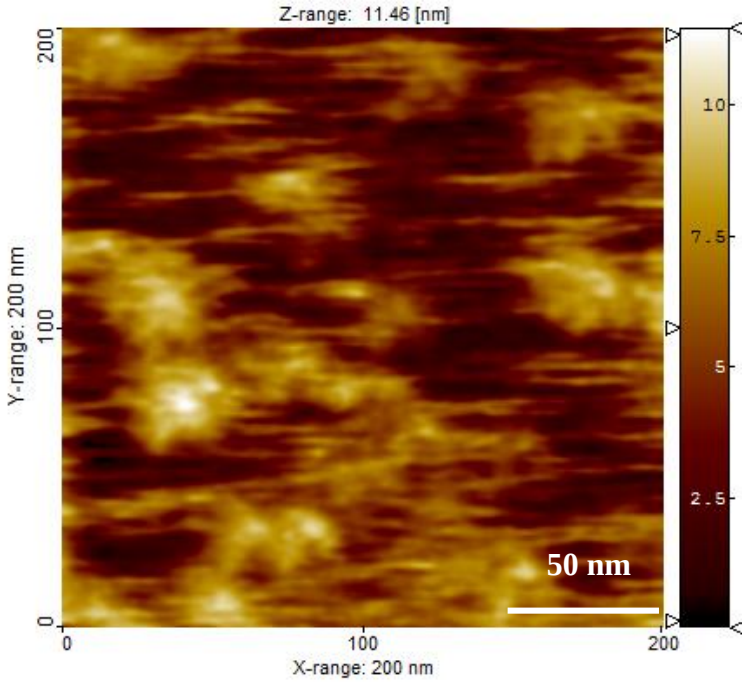

Fig. 2-1. AFM topography images of cellulase

Cellulases are comprised of a number of enzyme systems such as endoglucanase, exoglucanase and β-glucosidase. Three major groups of enzymes are involved in hydrolysis of cellulose to glucose and their action is synergistic. Endo-glucanase attacks regions of low crystallinity in the cellulose fiber and creates free chain-ends. Exo-glucanase degrades the molecule further by removing cellobiose units from the free

chain-ends which is then cleaved to glucose by the action of β-glucosidase. The enzymatihydrolysis can be influenced by substrate and end-product concentrations, enzyme activity and reaction conditions. β-Glucosidas plays a significant role in the hydrolysis process, since cellobiose an end-product inhibitor of many cellulases including both exo- and endo-glucanases [1-3]. β-Glucosidase, in turn, is inhibited by glucose and, enzymatic hydrolysis is thus sensitive to the substrate concentration.

2.1.2. Tetraethylorthosilicate (TEOS)

Sol-gel silica with excellent biocompatibility could be applied in immobilization of enzyme. Sol-gel silica were formed by hydrolysis and condensation of TEOS using acid or base catalyst [4, 5]. The TEOS structure is shown in Fig. 2-2.

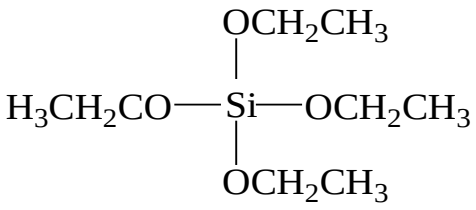

Fig. 2-2. Structures of TEOS

2.1.3. 1,1- diphenylethylene (DPE)

DPE were purchased from NacalaI Tesque, Inc, Kyoto, Japan. DPE is also one of the living radical polymerization approaches based on DPE as radical controlling agent. Generally, DPE is not a common monomer in the process of polymerization, and it is used as a chain transfer in radical polymerization [6-8]. Nevertheless, according to the copolymerization parameters of DPE, under acertain condition it can be copolymerization with other monomers. And in the process of copolymerization, DPE can stabilized the radical by the conjugate between the radical and the two phenyls. The DPE structure is shown in Fig. 2-3. So the DPE has an effect of controlled radicals in the polymerization. The use of DPE in conventional free radical polymerization allows a high degree of polymer structural control.

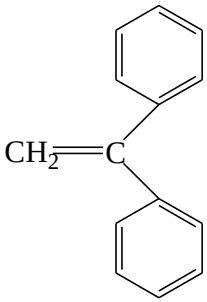

Fig. 2-3. Structures of DPE

2.2. Experiment method

2.2.1. Synthesis of magnetic supports

(a). Magnetite (Fe_3O_4) nanoparticles

Magnetite nanoparticles were prepared by the chemical coprecipitation method [8]. Fe^{2+} and Fe^{3+} in a molar ratio of 2:3 were mixed under flowing nitrogen gas with vigorous mechanical stirring at 80 °C. 28% $NH_3 \cdot H_2O$ was then added to the solution. The color of the bulk solution turned from orange to black immediately. The reaction was carried out at 80 °C for 2 h. The Fe_3O_4 nanoparticles were washed ten times with distilled water and then dried at 50 °C under vacuum for 12 h.

(b). Silica-coated magnetic nanoparticles Fe_3O_4/SiO_x

Coating the Fe_3O_4 nanoparticles by silica was carried out in a basic alcohol and water mixture at room temperature following the literature method [9]. 2 g Fe_3O_4 was dispersed in 40 mL distilled water and 160 mL alcohol by ultrasonic vibration for 1 h, then 5 mL $NH_3 \cdot H_2O$ and 5 mL TEOS were added to the reaction system, then the mixture was continuously mechanically stirred for 12 h. The resulting particles were washed ten times with distilled water and then dried at 50 °C under vacuum for 24 h. The obtained silica-coated magnetite nanoparticles are denoted as Fe_3O_4/SiO_x.

(c). Functionalized magnetic silica nanospheres (FMS)

Fe_3O_4/SiO_x (containing 1 g Fe_3O_4) was added to 60 mL alcohol. The solution was treated by ultrasonic vibration for 30 min. 6 mL $NH_3 \cdot H_2O$ and 4 mL APTES were added to the mixture and then temperature was increased to 50 °C under flowing nitrogen gas

[10]. The mixture was mechanically stirred for 8 h. The resulted FMS nanospheres was washed ten times with distilled water and were dried at 50 °C under vacuum for 24 h.

(d). Synthesis of magnetic composite microspheres

0.5 g Fe_3O_4, certain amount of KPS, ethanol (44 mL) and distill water (10 mL) was put into a flask and vibrated with ultrasonic for 1 h. Then the flask was placed in a water bath at 50 °C under nitrogen atmosphere. A mixture of 4 mL GMA, 4 mL EGDMA, 5.1 mL MATAC, DPE, ethanol and H_2O were dropwise into the flask in 30 min. Then the reaction was carried out at 50 °C for 2 h, 60 °C for 2 h, 75 °C for 2 h. The magnetic composite microspheres were isolated from the solution by a magnet, and then washed several time with ethanol.

2.2.2. Immobilization of cellulase on magnetic supports

(a). Immobilization of cellulase with cross-linking agent

The FMS nanospheres were activated using glutaraldehyde. FMS nanospheres (containing 1 g Fe_3O_4) were suspended in 8% 100 mL glutaraldehyde solution. The FMS activation was allowed to proceed at room temperature for 2 h.

Activated FMS nanospheres (containing 0.25 g Fe_3O_4) were put into cellulase solution, which was prepared from 0.1 g cellulase and 25 mL acetate buffer solution (pH 5.0). Activated FMS nanospheres were incubated at room temperature for 6 h. The resulting immobilized cellulase was stored at 4 °C until used.

(b). Immobilization of cellulase by physical absorption

Functionalized magnetic nanospheres (containing 0.25 g Fe_3O_4) were put into 4 mg/mL cellulase solution (pH 5.0) and were incubated at room temperature for 24 h. The resulting immobilized cellulase was washed three times with distilled water.

(c). Immobilization of cellulase by physical absorption and covalent bonds

Magnetic composite microspheres were put into 4 mg/mL cellulase solution (pH 5.0, acetate buffer solution) and were incubated at room temperature for 6 h. The resulting immobilized cellulase was washed several times with distilled water.

2.2.3. Cellulase activity assay

Activities of free and immobilized cellulase were determined using CMC as a substrate. The amount of glucose resulting from the hydrolysis of CMC substrate was

measured using the literature procedure [11]. 20 μL free cellulase or 10 mg immobilized cellulase has been incubated for 30 min with 1% CMC substrate in 1 mL acetate buffer solution [12]. The reducing sugars were determined by DNS agent. One unit of cellulase activity is defined as the amount of cellulase producing 1 μmol of glucose equivalents per minute. The unit was calculated as follows：

$$\text{Activity of cellulase}(\mu\text{mol} \cdot \text{mL}^{-1} \cdot \text{min}^{-1}) = \frac{1000W}{MVt}$$

where *W* is the amount of released glucose equivalents (mg), *M* is the molecular weight of glucose, *V* is the volume of the measured sample (mL) and *t* is the reaction time (min).

The relative activity (%) was the ratio between the activity of every sample and the maximum activity of sample.

The activity recovery (%) is the ratio between total cellulase activity in immobilized cellulase and total cellulase activity used for magnetic immobilized cellulase.

2.2.4. Determination loading efficiency of cellulase

The amount of cellulase in the supernatant before and after immobilization were determined by the Bradford method [13].

$$\text{Loading efficiency (\%)} = \frac{C_0V_0 - C_tV_t}{C_0V_0} \times 100$$

Here C_0 is the initial cellulase concentration (mg/mL), V_0 the initial volume of cellulase solution (mL), C_t is the cellulase concentration in the total supernatant (mg/mL), and V_t is total the cellulase volume (mL).

2.2.5. pH and temperature profiles of free and immobilized cellulase

pH and temperature profiles have been carried out in the same experimental conditions, excepting the studied parameter which was set at a proper value. pH profile was measured at 50 °C for 1 mL 0.1 M increments of buffer solution (pH 3.0-7.0) containing of 1% CMC substrate. The effect of temperature on enzyme activity was determined in 1 mL 0.1 M acetate buffer solution (pH 5.0) containing 1% CMC substrate for temperature from 30 °C to 70 °C, followed by the addition of 20 μL free cellulase or 10 mg immobilized cellulase and incubation for 30 min. Then, the relative activities were calculated by the ratio between the activity of each sample and the maximum activity of all samples.

2.2.6. Thermal stability of immobilized cellulase

Thermal stabilities of the free and immobilized cellulase on support were studied by measuring the residual activities of the cellulase after incubation in 0.1 M acetate buffer solution (pH 5.0) at 70 °C with continuous stirring. Samples were taken out at different time intervals and assayed for enzymatic activity. Relative activity was calculated as mentioned above.

2.2.7. Reusability of immobilized cellulase

Reusability of immobilized cellulase was examined by hydrolysis of CMC by the recovered immobilized cellulase with magnetic separation and compared with the value of first run (activity defined as 100%). Then activity of immobilized cellulase was determined.

2.3. Characterization of magnetic supports

2.3.1. Transmission electron microscopy (TEM)

The morphologies of magnetic supports prepared by reaction with different amounts of TEOS were characterized by transmission electron microscopy (TEM; Hitachi H-8100, Tokyo, Japan). For TEM observations, samples were dispersed in ethanol and then a small drop of the suspension was spread onto a 400 mesh copper grid.

2.3.2. Scanning electron microscope (SEM)

The morphologies of magnetic composite microspheres were characterized by Scanning electron microscope (SEM; S-4300, Hitachi Co., Ltd., Tokyo, Japan). The magnetic composite microspheres was sputter-coated by gold with Ion sputter (E-1030, Hitachi Co., Ltd., Tokyo, Japan) for 2 min to provide enhanced conductivity. The test voltage was 15 KV, and electric current was 10 μA.

2.3.3. Thermal analysis (TA)

Magnetic supports were examined by thermogravimetric analysis (TA) which was carried out in a Shimadzu TGA 50 analyzer equipped with an aluminum tube; samples were heated from room temperature to 600 °C with a scanning rate of 10 °C/min, whereas the flow of nitrogen was maintained at 50 mL/min. TGA test machine is shown in Fig. 2-4.

Fig. 2-4 TGA test machine

2.3.4. Fourier transforms infrared spectroscopy (FTIR)

The infrared spectra were measured using a micro sampling Fourier transforms infrared spectroscopy (FT-IR; MFT-2000, Jasco Co., Ltd., Tokyo, Japan) with the KBr pellet method. The wavelength range was between 4000 and 600 cm^{-1}, and the resolution was 4 cm^{-1}. Fifty scans were averaged for each sample.

2.3.5. Zeta potential measurements

Zeta potential measurements were conducted at pH 3-7 using in 1 mM KCl solution by Zeta potential analyzer (Brookhaven Instruments Corporation, USA).

2.3.6. X-ray diffraction (XRD)

X-ray diffraction (XRD) patterns were recorded in range of 2θ=5-90° by step scanning with a diffractometer (XRD-6000, Shimadzu Co., Ltd., Kyoto, Japan). Nickel-filter Cu Kα radiation (λ=0.15417 nm) was used with a generator voltage of 40 kV and a current of 30 mA. XRD test machine is shown in Fig. 2-5.

Fig. 2-5 XRD test machine

2.3.7. Vibrating-sample magnetometry (VSM)

The magnetic properties of magnetic supports were characterized with a vibrating sample magnetometer (VSM; Riken Denshi Co., Ltd., Tokyo, Japan).

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Chapter 3 Preparation of functionalized magnetic silica nanospheres for the cellulase immobilization

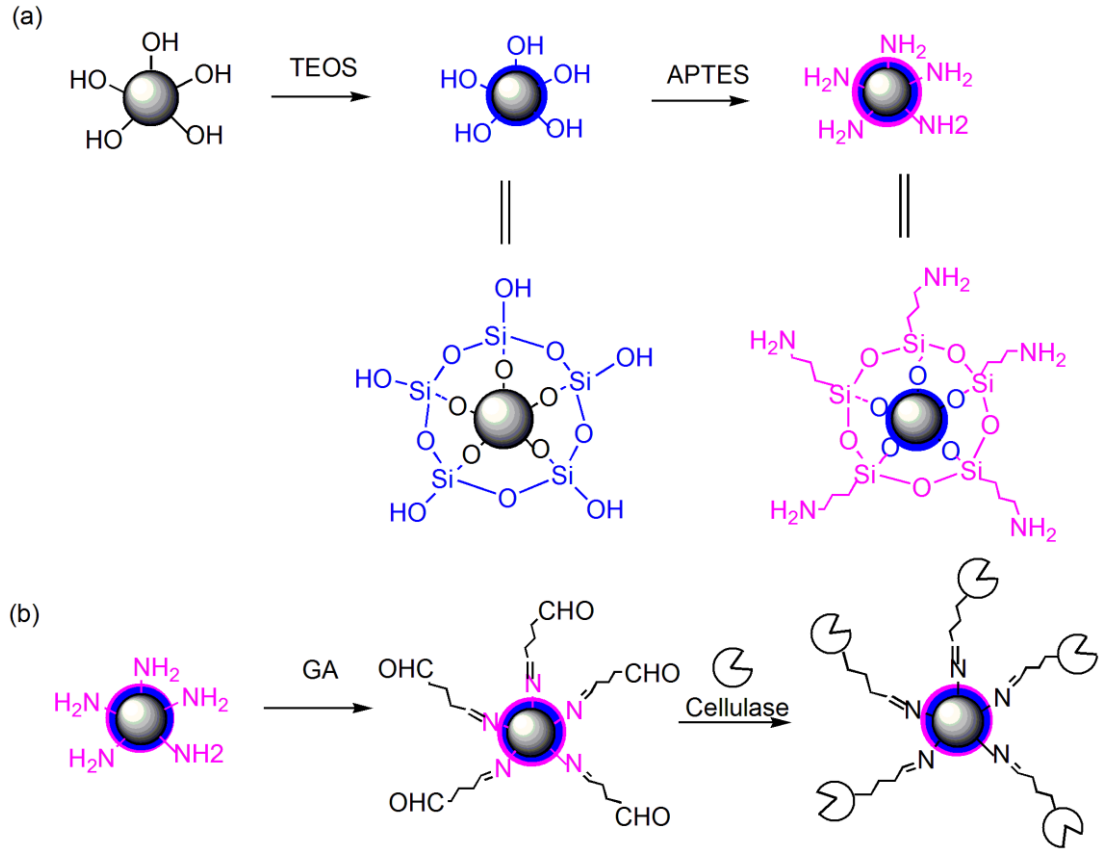
3.1. Introduction

Lignocellulosic complex is the most abundant organic materials found on the earth, and the use of lignocellulosic complex as a feedstock for the production of ethanol is a promising approach to meeting current and future energy demands in a sustainable way [1]. Bioethanol produced from lignocellulosic biomass is able to replace fossil fuel, and at the same time reduce the environmental pollution [2].

Conversion of lignocellulosic materials to bioethanol is a complex process, which requires improved cellulose and hemicelluloses conversion to reducing sugar by using cellulase [3]. Cellulases are comprised of a number of enzyme systems such as endoglucanase, exoglucanase and β-glucosidase. While endo- and exoglucanases are responsible for disrupting the cellulosic matrix, β-glucosidase converts the cellobiose, an intermediate product generated during endo-exo synergism, to reducing sugars. Enzymes are biocatalysts have certain desirable properties including high activity, selectivity and specificity. These properties facilitate the carrying out of complex chemical processes [4]. Nevertheless, use of cellulase to hydrolyze cellulose in a practical process has not been reported. The reasons are that cellulase is often easily inactivated and it is difficult to separate from reaction mixtures, which limits industrial application. Therefore, the important target in developing effective strategies for biofuel production is the complete use of the biocatalytic potential of the added cellulase.

Magnetic nanoparticles are important material for a wide range of application, such as bioseparation [5], biocatalysis [6], virus detection [7-9] and biomolecules immobilization [10,11], which was due to the ease of separating biomolecules from reaction solutions, their recyclability and preventing of biomolecules dissociation [12-14]. Coating magnetic nanoparticles with silica is becoming an important approach in the development of magnetic nanoparticles for both fundamental studies and technology applications. Silica deposited on the surface of magnetic nanoparticles can screen the magnetic dipolar attraction between magnetic nanoparticles, which favors the

dispersion of magnetic nanoparticles in liquid media and protects them from leaching in an acidic environment.



Scheme 3-1. (a) Synthesis of functionalized magnetic silica nanospheres and (b) immobilization of cellulase

Silica-coated magnetic nanoparticles can be easily activated to provide their surfaces with silanol groups [15-18]. Furthermore, functionalized magnetic silica spheres have been reported to exhibit high affinity for binding cellulase with amino-groups [19-24], the attachment of enzymes onto solid support through covalent bonds is a commonly used method for immobilization of enzymes. In addition, it can provide distinct advantages, such as the prevention of enzyme leakage from the support and improvement of the enzyme stability through point covalent attachment. However, silica content of coating on magnetite (Fe_3O_4) influences the formation of functionalized magnetic silica nanospheres and cellulase immobilization on this support. There are only a few reports about functionalized magnetic silica nanospheres being used as

supports for immobilization of cellulase to produce bioethanol.

In this study, synthesis and characterization of amino-silane modified silica nanospheres were performed. Fe_3O_4 nanoparticles were prepared by coprecipitation of Fe^{2+} and Fe^{3+} with NH_4OH, and then different amounts of tetraethylorthosilicate (TEOS) were used to coat silica on the surfaces of the Fe_3O_4 particles and form well-dispersed silica-coated magnetite particles. Amino-silane (APTES) was coupled to the surfaces of the magnetic silica nanospheres and immobilization of cellulase on the functionalized magnetic silica nanospheres using glutaraldehyde (GA) was carried out (Scheme. 3-1). The optimal conditions for preparation of functionalized magnetic silica nanospheres for the immobilization of cellulase and the stability of immobilized cellulase were also determined.

3.2. Materials and methods

3.2.1. Materials

Acremonium cellulase was purchased from Meiji Seika Pharma Co., Ltd., Tokyo, Japan., Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), glutaraldehyde (GA; 25%), carboxymethyl cellulose sodium salt (CMC), tetraethylorthosilicate (TEOS), were all extra pure reagent grade and purchased from Nacalai Tesque, Inc., Kyoto, Japan. The aminosilane coupling agents (APTES, AEAPTES and AEAPTMES) 3-aminopropyltriethoxysilane (APTES) was purchased from Shin-etsu chemical Co., Ltd.. 3.5-dinitrosalicylic acid (DNS) were obtained from Nacalai Tesque, Inc.

3.2.2. Synthesis of functionalized magnetic silica (FMS) nanospheres

Magnetite (Fe_3O_4) nanoparticles were prepared by the chemical coprecipitation method [25]. Fe^{2+} and Fe^{3+} in a molar ratio of 2:3 were mixed under flowing nitrogen gas with vigorous mechanical stirring at 80 °C. 28% $NH_3 \cdot H_2O$ was then added to the solution. The color of the bulk solution turned from orange to black immediately. The reaction was carried out at 80 °C for 2 h. The Fe_3O_4 nanoparticles were washed ten times with distilled water and then dried at 50 °C under vacuum for 12 h.

Coating the Fe_3O_4 nanoparticles with silica was carried out in a basic alcohol and water mixture at room temperature following the literature method [26]. 2 g Fe_3O_4 was dispersed in 40 mL water and 160 mL alcohol by ultrasonic vibration for 1 h, then 5 mL

$NH_3 \cdot H_2O$ and certain amount of TEOS were added to the mixture which was continuously mechanically stirred for 12 h. The resulting particles were washed ten times with distilled water and then dried at 50 °C under vacuum for 24 h. The obtained silica-coated magnetite nanoparticles are denoted as Fe_3O_4/SiO_x in this paper.

Next, Fe_3O_4/SiO_x (containing 1 g Fe_3O_4) was added to 60 mL alcohol. The solution was treated by ultrasonic vibration for 30 min. 6 mL $NH_3 \cdot H_2O$ and 4 mL APTES were added to the mixture and then temperature was increased to 50 °C under flowing nitrogen gas [27]. The mixture was mechanically stirred for 8 h. The resulted FMS nanospheres was washed ten times with distilled water and were dried at 50 °C under vacuum for 24 h.

3.2.3. Immobilization of cellulase on FMS nanospheres

The FMS nanospheres were activated using glutaraldehyde. FMS nanospheres (containing 1 g Fe_3O_4) were suspended in 8% 100 mL glutaraldehyde solution. FMS activation was allowed to proceed at room temperature for 2 h.

Activated FMS nanospheres (containing 0.25 g Fe_3O_4) were put into cellulase solution, which was prepared from 0.1 g cellulase and 25 mL acetate buffer solution (pH 5.0). Activated FMS nanospheres were incubated at room temperature for 6 h. The resulting immobilized cellulase was stored at 4 °C until used.

3.2.4. Characterization of Fe_3O_4/SiO_x and FMS nanospheres

(a). Characterization of Fe_3O_4/SiO_x prepared with different amounts of TEOS

The morphologies of Fe_3O_4/SiO_x prepared by reaction with different amounts of TEOS were characterized by transmission electron microscopy (TEM; Hitachi H-8100, Tokyo, Japan). For TEM observations, samples were dispersed in ethanol and then a small drop of the suspension was spread onto a 400 mesh copper grid. Fe_3O_4/SiO_x were examined by thermogravimetric analysis (TGA) which was carried out in a Shimadzu TGA 50 analyzer equipped with an aluminum tube; samples were heated from room temperature to 600 °C with a scanning rate of 10 °C/min in flowing nitrogen gas. The magnetic properties of Fe_3O_4/SiO_x were characterized with a vibrating sample magnetometer (VSM; Riken Denshi Co., Ltd., Tokyo, Japan).

(b). Characterization of FMS nanospheres

The morphologies of FMS nanospheres were characterized by transmission electron

microscopy (TEM; Hitachi H-8100, Tokyo, Japan). Structures of FMS nanospheres were studied by Fourier transform infrared spectroscopy (FT-IR), differential thermal analysis (DTA) and X-ray diffraction (XRD). The FT-IR spectra of FMS nanospheres were acquired with a MFT-2000 (Jasco, Tokyo, Japan) using the conventional KBr pellet method. DTA was carried out in a Shimadzu TGA 50 analyzer. Measurement conditions of DTA were the same as for TGA. The crystal structures of FMS nanospheres were investigated from the XRD patterns recorded in a range of 10-90° (2θ) Panalytical X´Pert Pro, (Cambridge, UK) with nickel-filter Cu Kα radiation (λ=0.15418 nm) of 40 kV voltage and 30 mA current.

3.2.5 Measurement of activities of free cellulase and immobilized cellulase

Activities of free and immobilized cellulase were determined using CMC as a substrate. The amount of glucose resulting from the hydrolysis of CMC substrate was measured using the literature procedure [28]. 20 μL free cellulase or 10 mg immobilized cellulase has been incubated for 30 min with 1% CMC substrate in 1 mL acetate buffer solution [29]. The reducing sugars were determined by DNS agent. One unit of cellulase activity is defined as the amount of cellulase producing 1 μmol of glucose equivalents per minute. The unit was calculated as follows：

$$\text{Activity of cellulase}(\mu\text{mol}\cdot\text{mL}^{-1}\cdot\text{min}^{-1}) = \frac{1000W}{MVt} \quad (1)$$

where W is the amount of released glucose equivalents (mg), M is the molecular weight of glucose, V is the volume of the measured sample (mL) and t is the reaction time (min).

The relative activity (%) was the ratio between the activity of every sample and the maximum activity of sample.

The activity recovery (%) is the ratio between total cellulase activity in immobilized cellulase and total cellulase activity used for magnetic immobilized cellulase.

3.2.6. Determination loading efficiency of cellulase

The amount of cellulase in the supernatant before and after immobilization was determined by the Bradford method [30].

$$\text{Loading efficiency}\ (\%) = \frac{C_0V_0 - C_tV_t}{C_0V_0} \times 100 \quad (2)$$

Here C_0 is the initial cellulase concentration (mg/mL), V_0 the initial volume of cellulase

solution (mL), C_t is the cellulase concentration in the total supernatant (mg/mL), and V_t is total the cellulase volume (mL).

3.2.7. Effect of the amount of TEOS used to prepare Fe_3O_4/SiO_x on the amount of immobilized cellulase.

The preparation method of Fe_3O_4/SiO_x described in Section 3.2.2 was followed, using TEOS amount of 1 mL, 3 mL, 5 mL and 7 mL. The obtained silica-coated magnetite particles were denoted as MS_1, MS_3, MS_5 and MS_7, respectively. Then FMS nanoparticles preparation was carried out for the four types of silica-coated magnetite nanoparticles. Then, these four preparations of FMS nanoparticles were used as support for immobilization of cellulase. The relative activity and the amount of immobilized cellulase and were detected by CMC method [28] and Bradford method [30], respectively.

3.2.8. Thermal stability of immobilized cellulase

Thermal stabilities of the free and immobilized cellulase on support were studied by measuring the residual activities of the cellulase after incubation in 0.1 M acetate buffer solution (pH 5.0) at 70 °C with continuous stirring. Samples were taken out at different time intervals and assayed for enzymatic activity. Relative activity was calculated as mentioned above.

3.2.9. Reusability of immobilized cellulase

Reusability of immobilized cellulase was examined by hydrolysis of CMC by the recovered immobilized cellulase with magnetic separation and compared with the value of first run (activity defined as 100%). Then activity of immobilized cellulase was determined.

3.3. Results and Discussion

3.3.1. Preparation and characterization of Fe_3O_4/SiO_x prepared with different amounts of TEOS

(a). Morphologies

TEM micrographs of Fe_3O_4/SiO_x prepared with different amounts of TEOS are shown in Fig. 3−1.

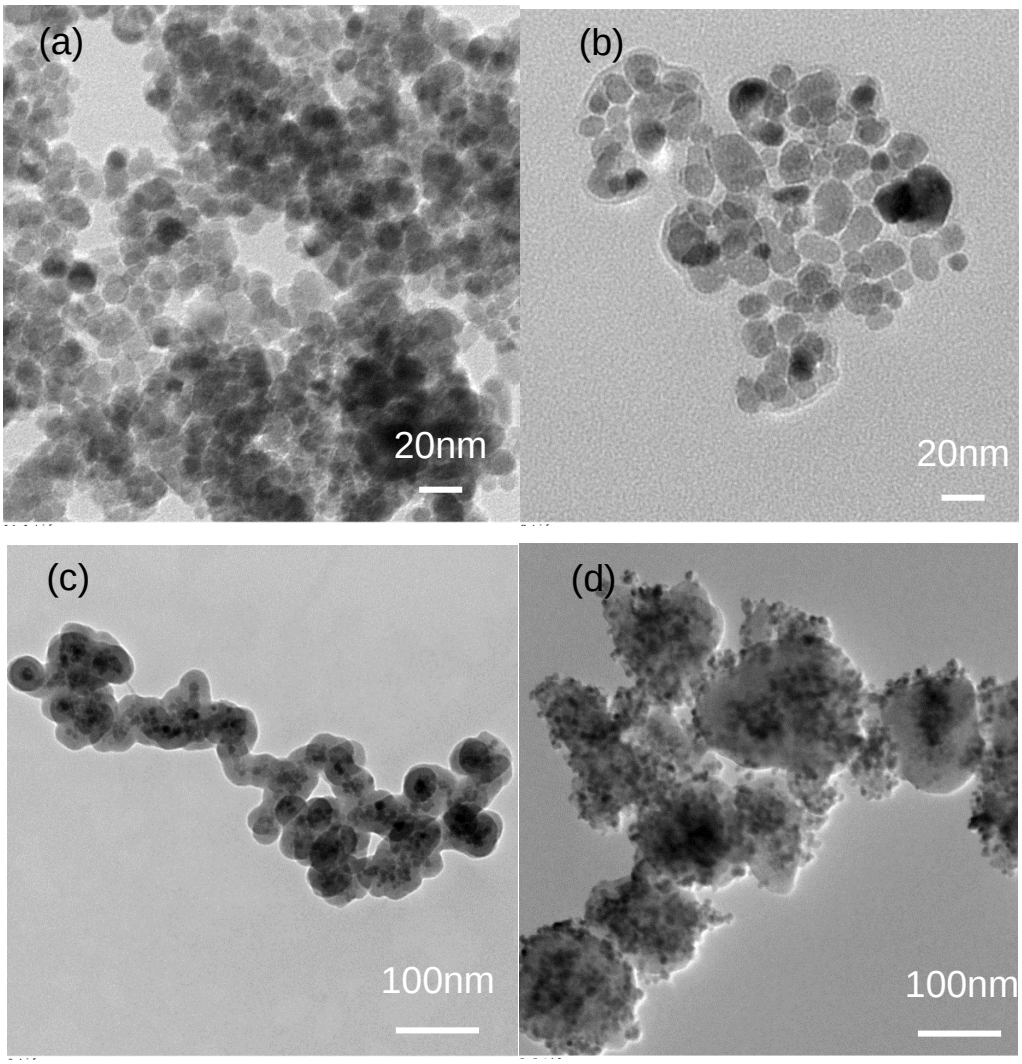


Fig. 3-1. TEM image of (a) Fe_3O_4 and Fe_3O_4/SiO_x prepared with different amounts of TEOS: (b) MS_3, (c) MS_5, and (d) MS_7.
(a, b) magnification 50 k, (c, d) magnification 20 k

The MS_3 micrograph (Fig. 3−1 (b)) reveals that a thin silica layer is deposited on Fe_3O_4 at room temperature, the agglomeration of the nanoparticle is broken, and the dispersity of nanoparticle is improved. The amount of TEOS influences the morphologies of the silica-coated magnetite nanoparticles. The content of silica coating on the surface of Fe_3O_4 gradually increases with the increasing the amount of TEOS from 1 mL to 5 mL. The MS_5 forms clear core-shell structure, which is due to silica coated on the surface of Fe_3O_4. However, for the reaction with a higher amount of TEOS (7 mL TEOS), silica was coated on aggregate magnetite particles, and excessive TEOS leads to the aggregate of magnetic nanospheres as shown in Fig. 3-1 (d). Hence,

reaction with 5 mL TEOS forms the silica-coated magnetite particles with better dispersion and more desirable morphologies than reaction with the other amounts of TEOS.

(b). Thermal analysis

In order to study the compositions in Fe_3O_4/SiO_x prepared with different amount of TEOS, TGA was carried out and results are shown in Fig. 3−2. The silica content of Fe_3O_4/SiO_x can be calculated according to weight loss of Fe_3O_4/SiO_x obtained by TGA testing, and the respective silica contents in MS_1, MS_3, MS_5 and MS_7 are 20%, 27%, 37% and 40%. The content of silica in Fe_3O_4/SiO_x gradually increases with the increase of the amount of TEOS. However, the silica content does not change significantly when the amount of TEOS is more than 5 mL, which might be due to TEOS deposited on aggregation Fe_3O_4 and an insufficient number of -OH groups on the Fe_3O_4 surface, so that most of the TEOS would not be hydrolyzed on surface of Fe_3O_4.

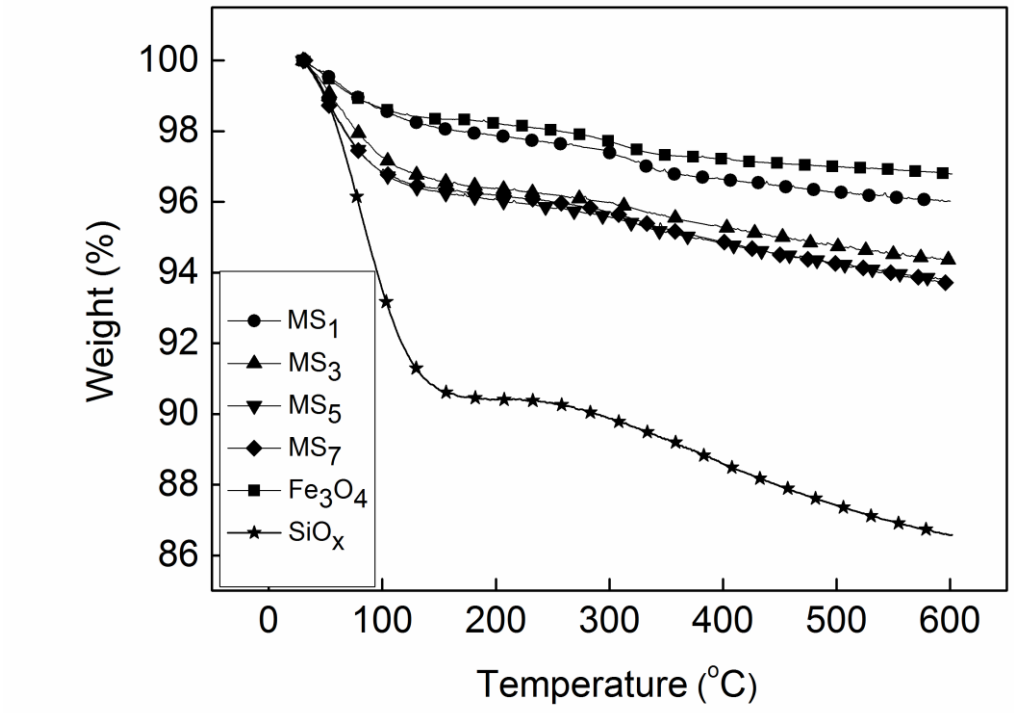


Fig. 3-2. TGA results for Fe_3O_4, SiO_x and Fe_3O_4/SiO_x prepared with different amounts of TEOS. Samples were heated from room temperature to 600 °C with a scanning rate of 10 °C/min in flowing nitrogen gas

(c). Magnetic behavior

To study the magnetic properties of magnetite nanoparticles before and after silica coating of the particles, the hysteresis loops of magnetite nanoparticles and the silica-coated magnetite nanoparticles with different silica contents were measured with VSM.

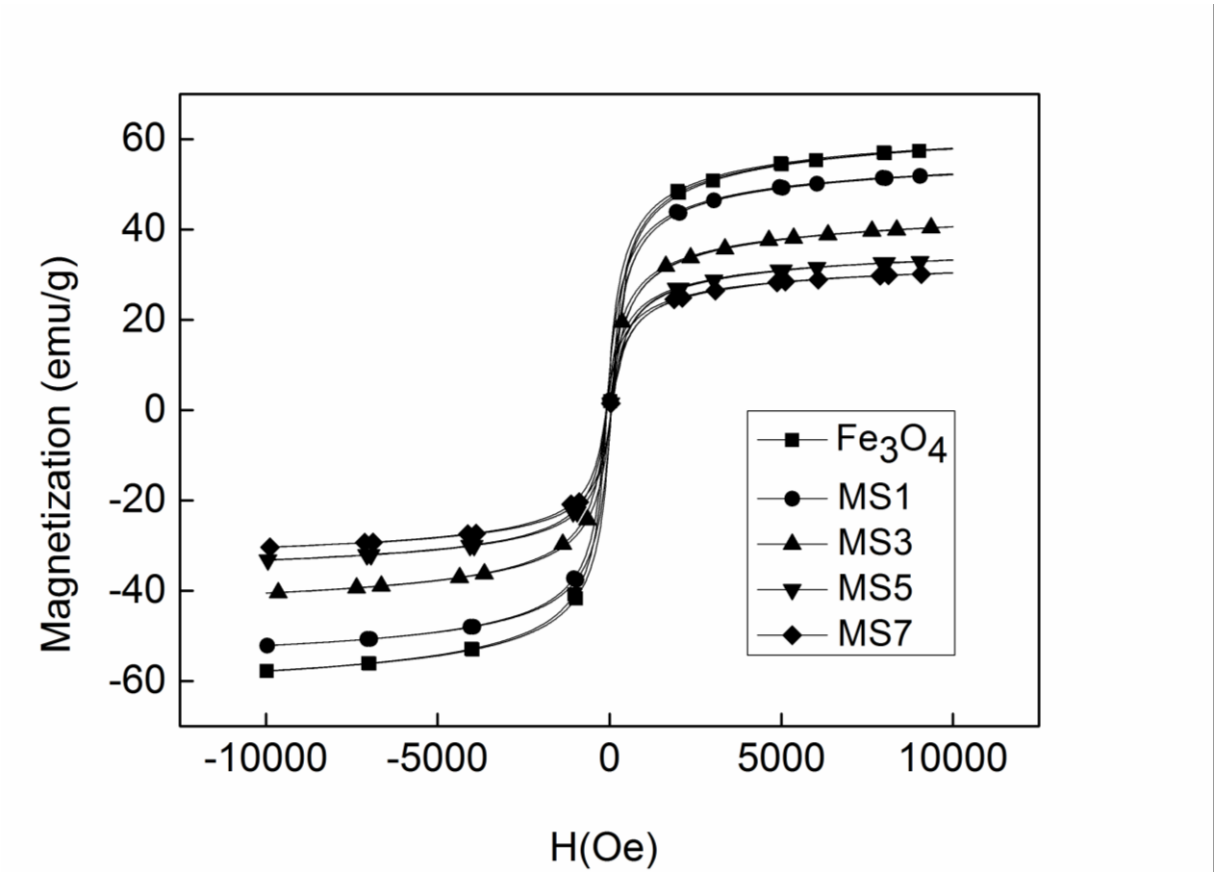


Fig. 3-3. Magnetic behaviors of Fe_3O_4 and Fe_3O_4/SiO_x prepared with different amounts of TEOS

The magnetic properties of the nanoparticles are significant for all further applications [31, 32]. Fig. 3-3 shows that the saturation magnetization of Fe_3O_4 and MS_5 were found to be 57.6 emu/g and 33 emu/g, respectively. This difference suggests that a thin silica layer was successfully deposited on the surface of magnetite particles. However, when the reaction media with the high concentration of TEOS (7 mL), the saturation magnetization of MS_7 was stable, which is in accordance with thermal analysis.

3.3.2. Characterization of immobilized cellulase

(a). Morphologies

TEM images of Fe_3O_4, FMS and FMS-GA-EN are shown in Fig. 3-4. The preparation of Fe_3O_4 (Fig. 3-4 (a)) was obtained by a chemical coprecipitation method from ferrous and ferric ion solutions with a molar ratio of 2:3. The average particles sizes of Fe_3O_4 ranged from 10-13 nm with extensive aggregation. Fe_3O_4 particles prepared by the coprecipitation method have quite a number of hydroxyl groups on their surface due to contact with the aqueous phase. These Fe_3O_4 particles tend to aggregate to form clusters. The TEM image of Fig. 3-4 (b) clearly shows the FMS form sphere-shaped morphologies that consist of a core (the dark area in the image) and shell (the light area), which is silica deposited on the surface of the Fe_3O_4 particles to form SiO_x coating layer. Serious aggregation of Fe_3O_4 nanoparticles is improved to some extent, because the silica coating on the magnetic nanoparticles screens the magnetic dipolar attraction between magnetic nanoparticles [33]. By comparing images of Fig. 3-4 (b) and (c), it is clear that the average particle size of FMS-GA-EN was larger than that of FMS, which might be due to cellulase was coupled on the FMS.

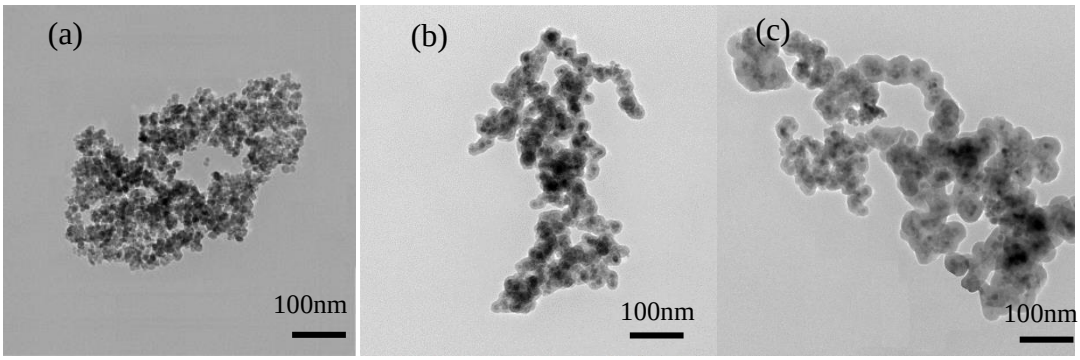


Fig. 3-4. TEM images of (a) Fe_3O_4, (b) FMS and (c) FMS-GA-EN. (Magnification 50 k)

(b). FT-IR analysis

FT-IR spectra of the Fe_3O_4, Fe_3O_4/SiO_x, FMS, FMS-GA, FMS-GA-EN and EN are reproduced in Fig. 3-5. The spectrum of Fe_3O_4 (Fig. 3-5 (a)) shows the stretching vibrations of –OH at 3423 cm^{-1}, which is assigned to OH^- absorbed by Fe_3O_4 particles. The peak at 621 cm^{-1} is attributed to the Fe-O bond vibration of Fe_3O_4. New peaks at

1091 cm^{-1} and 960 cm^{-1} are observed for Fe_3O_4/SiO_x (Fig. 3-5 (b)) and they are assigned to Si-O stretching of siloxane and the Si-OH bending, respectively. However, the intensity of the Fe-O bond vibration peak at 621 cm^{-1} decreases after the loading of silica and there is a little removal. These facts all confirm that the silica layer was coated successfully on the magnetite particles at room temperature. The FMS spectrum (Fig. 3-5 (c)) is characterized by the decreased intensity of the Si-O at 1091 cm^{-1} that appears for Fe_3O_4/SiO_x. After FMS activation by GA, the peak at 1746 cm^{-1} is assigned to the vibration of C=O in FMS-GA. After immobilization of cellulase on the supports, the product peaks at 1544 cm^{-1}, 1464 and 1400 cm^{-1} can be assigned to the heterocycle, methylene and/or methyl and other groups of cells, respectively. The support reacted with the amino group of cellulase to form the imide group, which indicates the binding of cellulase.

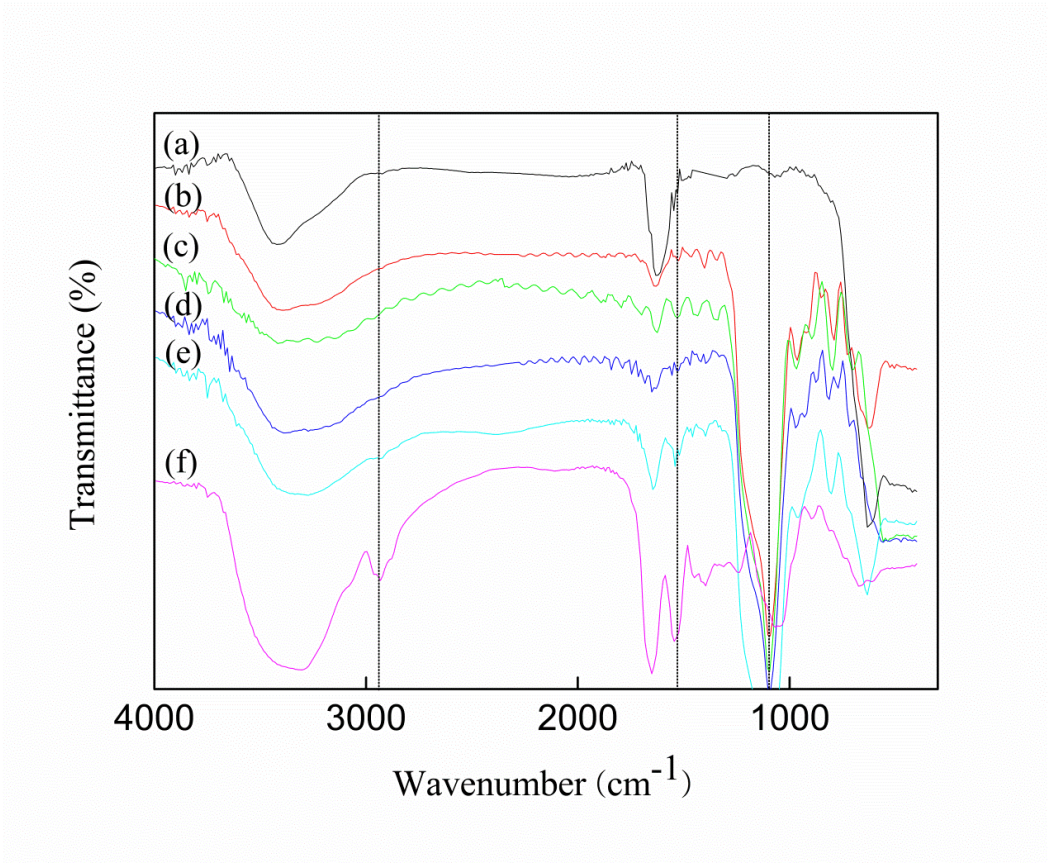


Fig. 3-5. FT-IR spectra of (a) Fe_3O_4, (b) Fe_3O_4/SiO_x, (c) FMS, (d) FMS-GA, (e) FMS-GA-EN and (f) EN. The FT-IR spectra of FMS nanospheres were investigated by the conventional KBr pellet method

(c). Thermal analysis

The thermal analysis of Fe_3O_4, Fe_3O_4/SiO_x (MS5), FMS, FMS-GA, FMS-GA-EN were investigated by DTA. The DTA curves (Fig. 3-6) show that the first thermal flow up to 145 °C is probably due to the evaporation of adsorbed water and hydration water. Then Fe_3O_4/SiO_x exhibits two new thermal flows: the thermal flow at about 200 °C comes from decomposition of organic silica in Fe_3O_4/SiO_x, and the other flow at about 340 °C is probable due to cracking of organic silica and the Fe_3O_4 surface. Moreover, FMS has a new thermal flow at 420 °C due to the decomposition of organic silicane in FMS. Hence, amino silane APTES were coupled to the surface of the magnetic silica nanospheres. After immobilization of cellulase, a new thermal flow at 450 °C for FMS-GA-EN. These findings indicate FMS are activitied by GA and cellulase was immobilized on FMS.

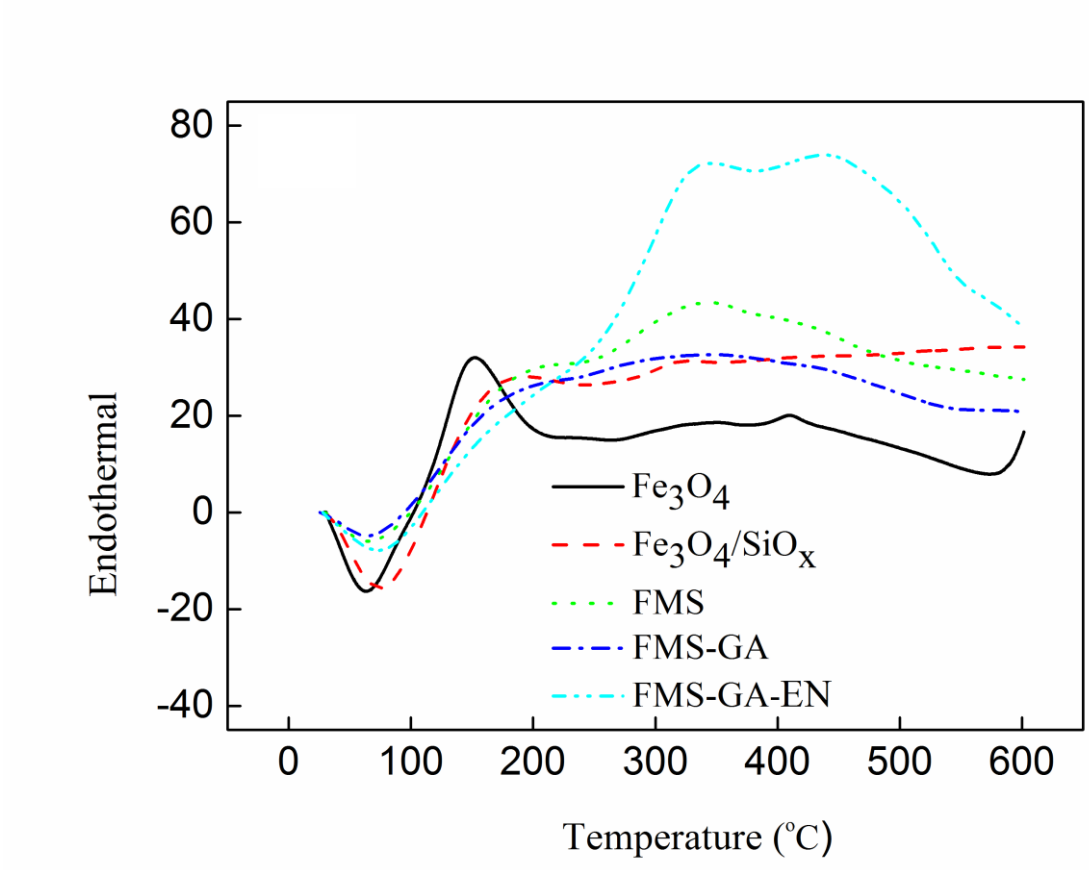


Fig. 3-6. DTA of Fe_3O_4 Fe_3O_4/SiO_x, FMS, FMS-GA and FMS-GA-EN. Samples were heated from room temperature to 600 °C with a scanning rate of 10 °C/min in flowing nitrogen gas

3.3.3. Optimum conditions of enzymatic activity

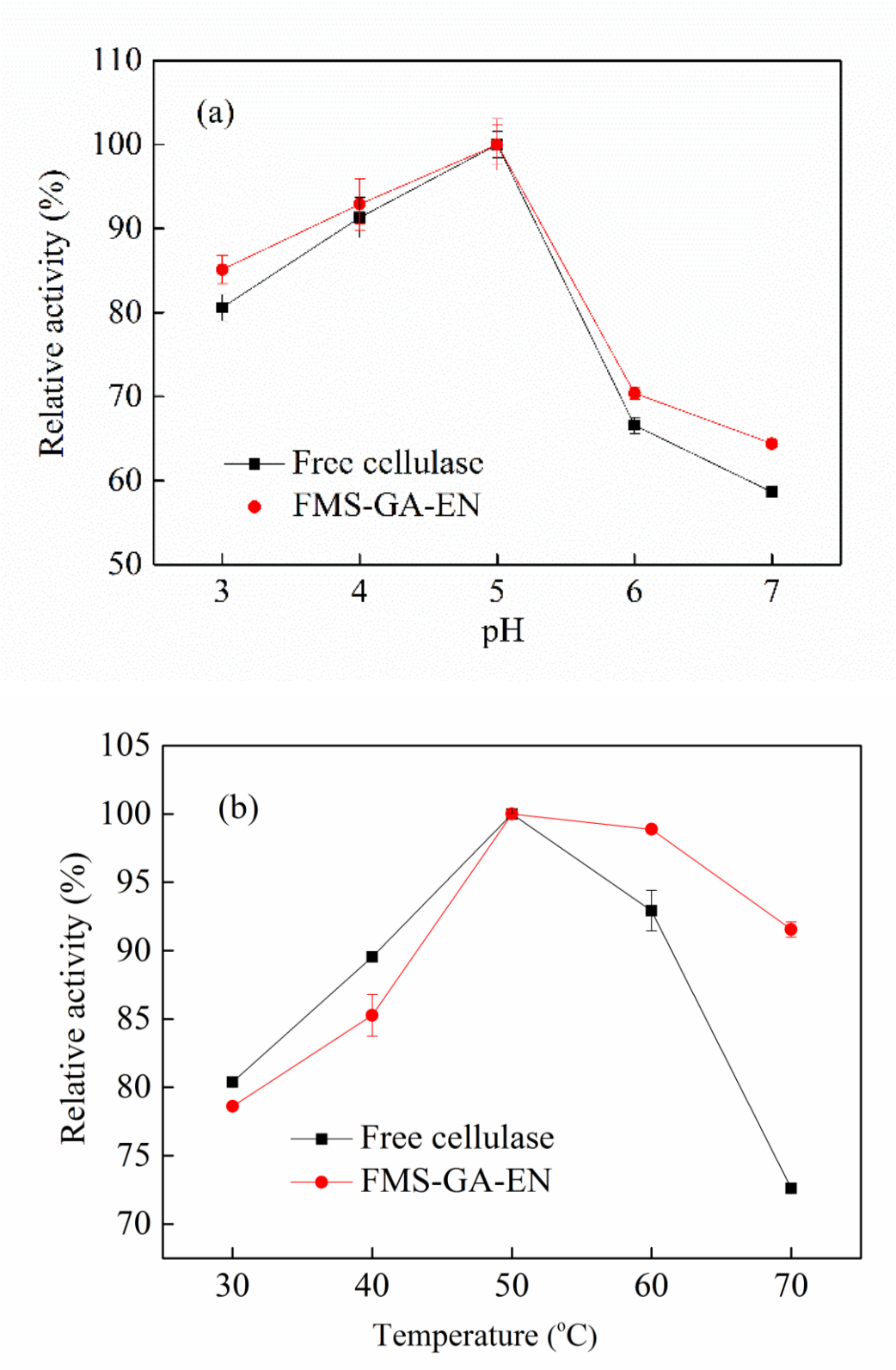


Fig. 3-7. Effects of pH and temperature on the activity of free cellulase and FMS-GA-EN: (a) pH and (b) temperature

The optimal pH following immobilization was shown in Fig. 3-7 (a). Both free cellulase and immobilized cellulase exhibit the maximal activity at pH 5.0. Fig. 3-7 (b) shows that free cellulase and immobilized cellulase exhibit thermal inactivation at high temperature; however, relative activity of immobilized cellulase during 50-70 °C interval is 10-20% higher than free cellulase. Immobilized cellulase has better pH and temperature stability due to the carriers enhancing the enzyme rigidity.

3.3.4. Thermal stabilities of free cellulase and immobilized cellulase

Thermal stabilities of free and immobilized cellulase were compared by measuring their activities at 70 °C in acetate buffer solution (pH 5.0). The data plotted in Fig. 3-8 demonstrates that immobilized and free cellulase lost activities as the time is increased. However, immobilized cellulase has better thermal resistance than the free cellulase, the similar behavior has also been observed in the other reports [34, 35]. The enhancement of thermal stability is likely attributed to covalent bonding between cellulase and amino groups supports.

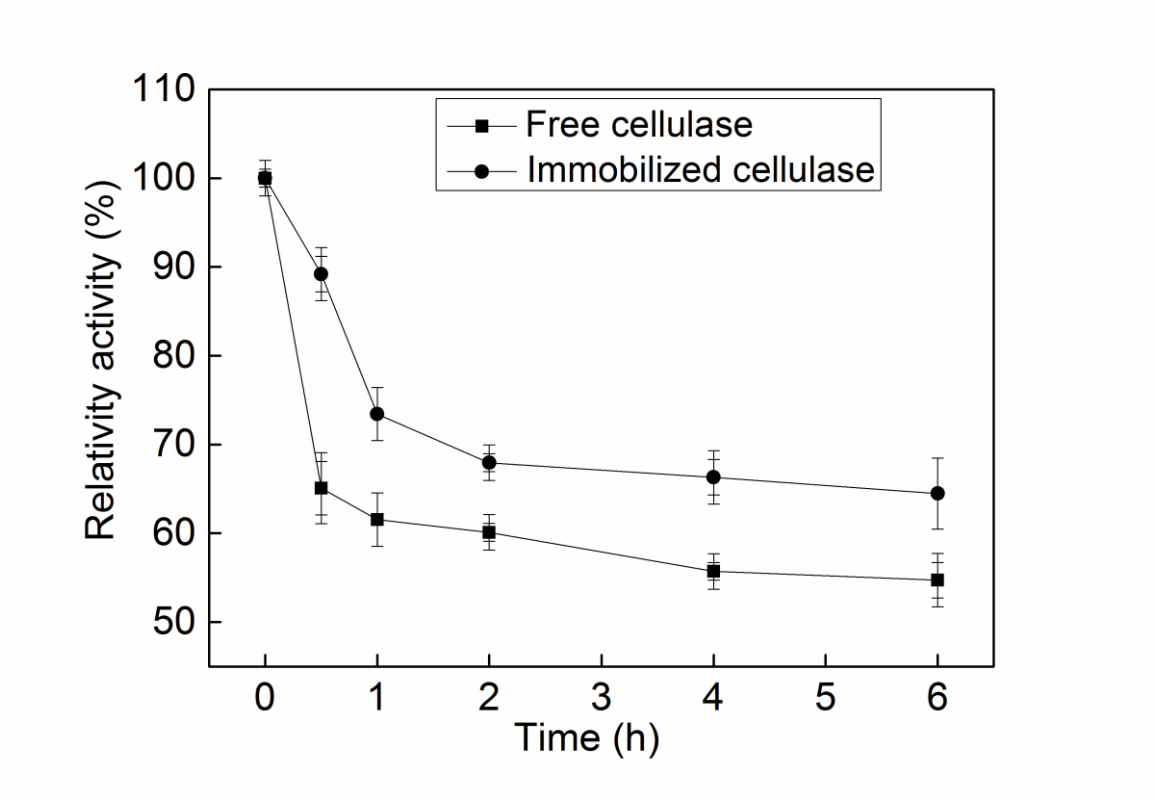


Fig. 3-8. Thermal stabilities of free and immobilized cellulase. CMC substrate was hydrolyzed by free cellulase and immobilized cellulase at 70 °C for 6 h

3.3.5. Reusability of immobilized cellulase

The reusability of immobilized cellulase is an important parameter in large-scale industrial application for economic reason. The reusability of immobilized cellulase was determined by the repeated use of the same biocatalyst for 10 reaction cycles and results are shown in Fig. 3-9. The activity of immobilized cellulase significantly decreased from reaction cycles from 1 to 4, the activity becomes stable after that. The decrease of the cellulase activity likely result from the eventual cellulase leakage, that is desorption of a very small amount of residue cellulase absorbed on the supports; but the immobilized cellulase does not become completely inactivated after 10 reaction cycles. Immobilized cellulase has significant stability and retains 85.5% of initial activity after 10 continuous uses. Thus the optimized preparation conditions for the FMS lead to immobilized cellulase with significantly higher stability and reusability for large-scale industrial application.

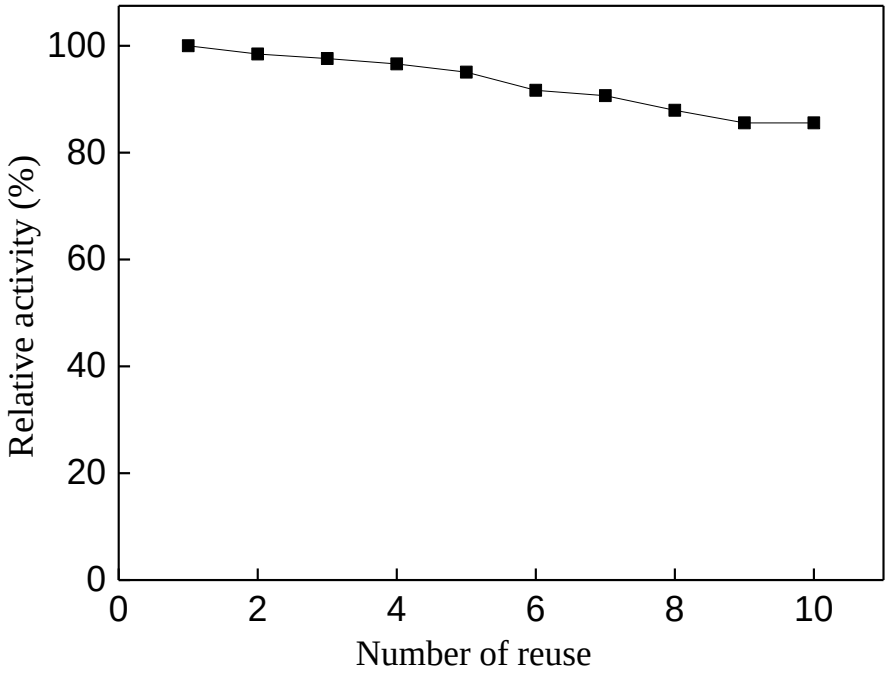


Fig. 3-9. Reusability of immobilized cellulase. CMC substrate was hydrolyzed by immobilized cellulase at 50 °C for 10 reaction cycles, each reaction time was 24 h

3.4. Conculsions

Silica was coated on Fe_3O_4 nanoparticles to form silica-coated magnetite nanoparticles. When the Fe_3O_4 nanoparticles were reacted with 5 mL of TEOS as the optimal amount, the MS_5 prepared with core-shell structure had 37% silica content and good dispersion. Amino silane was coupled to the surface of silica-coated magnetite nanoparticles, which was proved by FT-IR and DTA. Wide-angle X-ray diffraction pattern of FMS nanospheres showed that they had the spinel structure similar to Fe_3O_4.

The FMS nanospheres prepared with 5 mL of TEOS was used as support for immobilization of cellulase. The amount of immobilized cellulase was 92 mg/g support and the activity recovery was 80%. Immobilized cellulase exhibited better resistance to high temperature in comparison to free cellulase. The activity of the immobilized cellulase is still 63.2% retention of the initial activity after 10 continuous uses, which demonstrated the large-scale industrial applications.

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Chapter 4 Preparation of three amine-silane functionalized magnetic silica nanospheres for cellulase immobilization

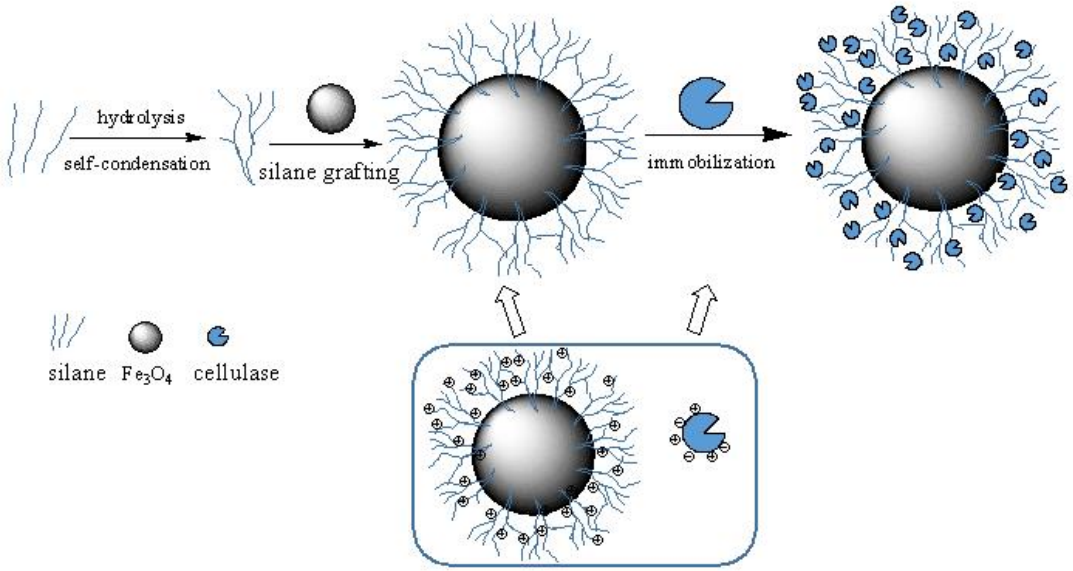
4.1. Introduction

Bioethanol from cellulosic biomass is one of the most promising biofuels to lessen the environmental pollution generated by burning of fossil fuels [1, 2]. Conversion of cellulosic materials to ethanol is a complex process. Initially, cellulose is hydrolyzed into reducing sugar by cellulase and reducing sugar is subsequently converted into bioethanol [3, 4]. These two steps can be performed in a combined hydrolysis and fermentation, referred to as simultaneous saccharification and fermentation (SSF), which is mainly used for bioethanol production [5-6]. Cellulase is a multicomponent enzyme consisting of three different enzymes (1,4-β-D-glucanases or endoglucanases, exo-1,4-β-D-glucanases or cellobiohydrolases and 1,4-β-D-glucosidases). Enzymes are biocatalysts possessing some excellent properties, such as of high activity, selectivity and specificity [7]. Nevertheless, the cellulase is often easily inactivated and difficult to be separated [8-9]. The optimal temperature for SSF is about 38 °C, which is a compromise between the optimal temperatures for hydrolysis (45-50 °C) and fermentation (30 °C). The efficiency of free cellulase is limited by their deactivation at about 38 °C [10]. Therefore, developing effective strategies for improving enzyme stability is an important target for ethanol production.

Immobilization of enzymes on supports in general helps to improve the enzyme stability by increasing the resilience of enzymes to variation of pH and temperature [11-14]. Immobilization of cellulase has been achieved using a diverse range of methodologies (adsorption on nanostructured materials and covalent binding of cellulase on nanomaterials) [15]. Covalent binding normally leads to the partial inactivation due to conformational restrictions caused by covalent binding of amino acid residues to support. Adsorption has the highest commercial potential, due to its relatively low cost, simplicity, and high retention of catalytic activity [16].

Magnetic particles are important material for a wide range of application, such as bioseparation [17], hyperthermia [18-20], and biomolecules immobilization. Fe_3O_4

nanoparticles have received extensive attention in enzyme immobilization to improve enzyme activity, loading and stability. Fe_3O_4 nanoparticles with low toxicity, biocompatibility, and easy synthesis are more suitable supports for enzyme as compared to others [21, 22]. Surface modification using organic functional groups has been found to be useful for the immobilization and adsorption of enzymes to the surface of the silica material [23, 24]. Magnetite nanoparticles coated with silica and modified by organic-silanes, biocompatible and with hydrophilic properties, are promising approach in nanobiotechnology. The huge diversity of alkoxysilanes allows for different types of nanoparticle surface functionalization, i.e., introducing charges on the system that can prevent the aggregation of the particles in liquids and improve their biocompatibility [25, 26]. Surface modification by silanes is a complex process [27, 28]: First, the silane monomers are hydrolyzed liberating alcohol and yielding reactive silanol groups. Second, during the hydrolysis process, the concomitant condensation of silanols (aging) also takes place. Finally, silanes are adsorbed and chemically grafted on magnetic silica nanospheres surfaces. However, few papers have reported on the functionalization of magnetic silica nanospheres is used as supports for immobilization of cellulase through the interface-containing amino groups. Silane structure is correlated to silane grafting on the surface of functionalization of magnetic silica nanospheres and immobilization of cellulase. So the control of grafted silane becomes an important step in this system.



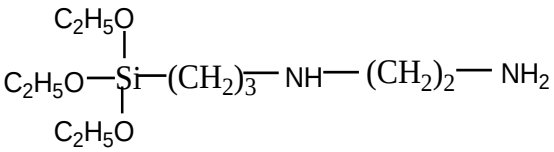
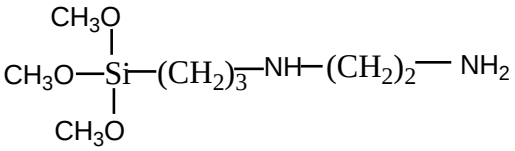
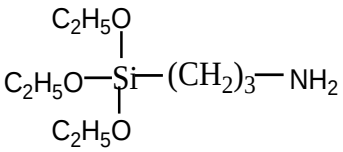
Scheme 4-1. Synthesis of functionalized magnetic silica nanospheres and immobilization of cellulase

In the present work, functionalized magnetic nanospheres were prepared by hydrolysis of amino-silane and grafting of amino-silane on the surface of unfunctionalized magnetic nanospheres, and then immobilization of cellulase on functionalized magnetic nanospheres was conducted by electrostatic adsorption. (Scheme 4-1). The influence of silane structure in alkoxysilanes containing the amino groups on modification of unfunctionalized magnetic nanospheres surface was studied by transmission electron microscopy (TEM), zeta potential analysis, thermogravimetric analysis (TGA), and vibrating sample magnetometry (VSM). The properties of immobilized cellulase on these functionalized magnetic nanospheres were investigated, including the amount of immobilized cellulase and its relative activity and stability.

4.2. Experimental method and characterization

4.2.1. Materials

Table 4-1. Amino-silane and their chemical structures

Silane	Chemical names	Chemical structures
AEAPTES	3-(2-aminoethyl aminopropyl)-trieth oxysilane	
AEAPTMES	3-(2-aminoethyl aminopropyl)-trime thoxysilane	
APTES	3-aminopropyl triethoxysilane	

The amino-silane coupling agents (APTES, AEAPTES and AEAPTMES) were purchased from Shin-Etsu Chemical Co., Ltd. Japan. The chemical structures and description are listed in Table 4-1.

4.2.2. Synthesis of functionalized magnetic nanospheres

Magnetite (Fe_3O_4) nanoparticles were prepared by the conventional chemical coprecipitation [29]. Silica coated nanospheres were prepared by the Stöber method [30].

First, 2 g magnetite particles were mixed with 40 mL water, 160 mL alcohol and 5 mL $NH_3 \cdot H_2O$ by ultrasonic vibration for 1 h, and then 5 mL TEOS was added in the mixture under continuous vigorous stirring for 12 h at room temperature, silica was formed on the surface of magnetite nanoparticles through hydrolysis and condensation of TEOS. The resulting particles unfunctionalized magnetic nanospheres were washed ten times with distilled water and then dried at 50 °C under vacuum for 24 h.

Unfunctionalized magnetic nanospheres (containing 1 g Fe_3O_4) were added to 60 mL alcohol. The solution was treated by ultrasonic vibration for 30 min at room temperature. 6 mL $NH_3 \cdot H_2O$ and 4 mL amino-silane (AEAPTES, AEAPTMES and APTES) were added into the mixture and then the temperature was increased to 50 °C under nitrogen gas with vigorous stirring for 8 h [21]. The magnetic nanoparticles were washed ten times with distilled water and dried at 50 °C under vacuum for 24 h. The resulted functionalized magnetic nanospheres were denoted as S1, S2 and S3, respectively.

4.2.3. Immobilization of cellulase

Functionalized magnetic nanospheres (containing 0.25 g Fe_3O_4) were put into 4 mg/mL cellulase solution (pH 5.0) and were incubated at room temperature for 24 h. The resulting immobilized cellulase was washed three times with distilled water.

4.3. Results and discussion

4.3.1. Characterization of functionalized magnetic nanospheres

(a). Morphologies

TEM micrographs of Fe_3O_4 nanoparticles and three functionalized magnetic nanospheres are shown in Fig. 4-1. The average particle sizes of Fe_3O_4 nanoparticles ranged from 10 to 13 nm with extensive aggregation, which attributed to the hydroxyl groups on their surface [34, 35]. The functionalized magnetic nanospheres micrographs reveal that functionalized magnetic nanospheres form sphere-shaped morphologies that consist of a core (the dark area in the image) and shell (the light area) [35]. Moreover, the agglomeration of the ultra-fine particles have been broken to some extent. The improved dispersion was caused by that silica was deposited on the surface of the Fe_3O_4 nanoparticles to form SiO_x coating layer and charges was introduced on the system to prevent the aggregation of Fe_3O_4 nanoparticles by silane. The improved dispersion can

enhance the efficiency of immobilized enzymes; for example, regarding the surface area for the attachment of enzymes, higher enzyme loading per unit mass of particles [36].

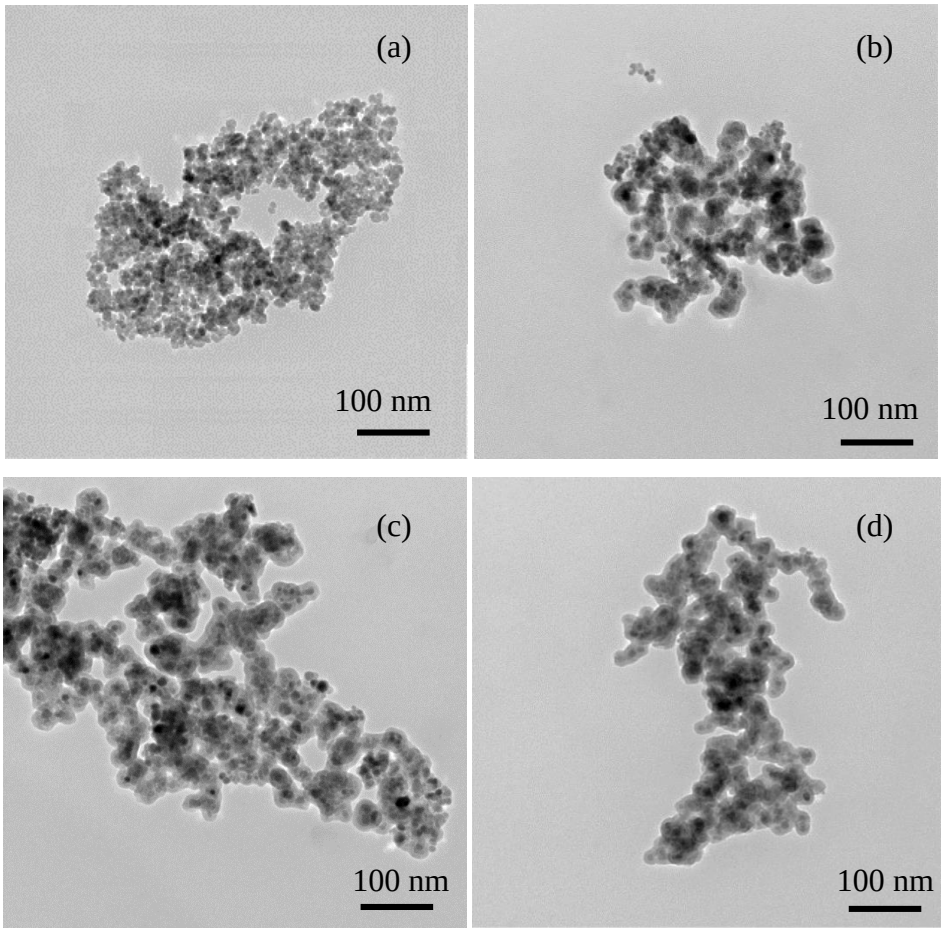


Fig. 4-1. TEM images of (a) Fe_3O_4 and three functionalized magnetic nanospheres: (b) S1, (c) S2 and (d) S3

(b). Surface electric charge determined by zeta potential

The zeta potential of functionalized magnetic nanospheres and unfunctionalized magnetic nanospheres were measured and the zeta potential of three functionalized magnetic nanospheres are shown in Fig. 4-2. Unfunctionalized magnetic nanospheres display the increase of negative zeta potential with increasing pH, which is caused by the enhanced adsorption of hydroxyl ions [37]. After silane treatment, the zeta potential of the negative surface of unfunctionalized magnetic nanospheres shifted to positive surface, which confirms that the modification process was effective through hydrolysis and grafting of silane. Moreover, the zeta potential of S2 (29 mV) is higher than S3 (25

mV), which agrees with previous data [38], and S1 (16 mV) at pH 5, which have been caused by the highest level of the amino group of AEAPTMES. The zeta potential of S3 is higher than S1 though AEAPTES has higher level of the amino group, which might be due to more silane grafted on unfunctionalized magnetic nanospheres surface. APTES have shorter alkyl spacer between the functionality and the silicon atom than AEAPTES. Shorter alkyl spacer causes higher reactivity of alkoxy group due to the stronger electron interaction between the functionality and the silicon atom.

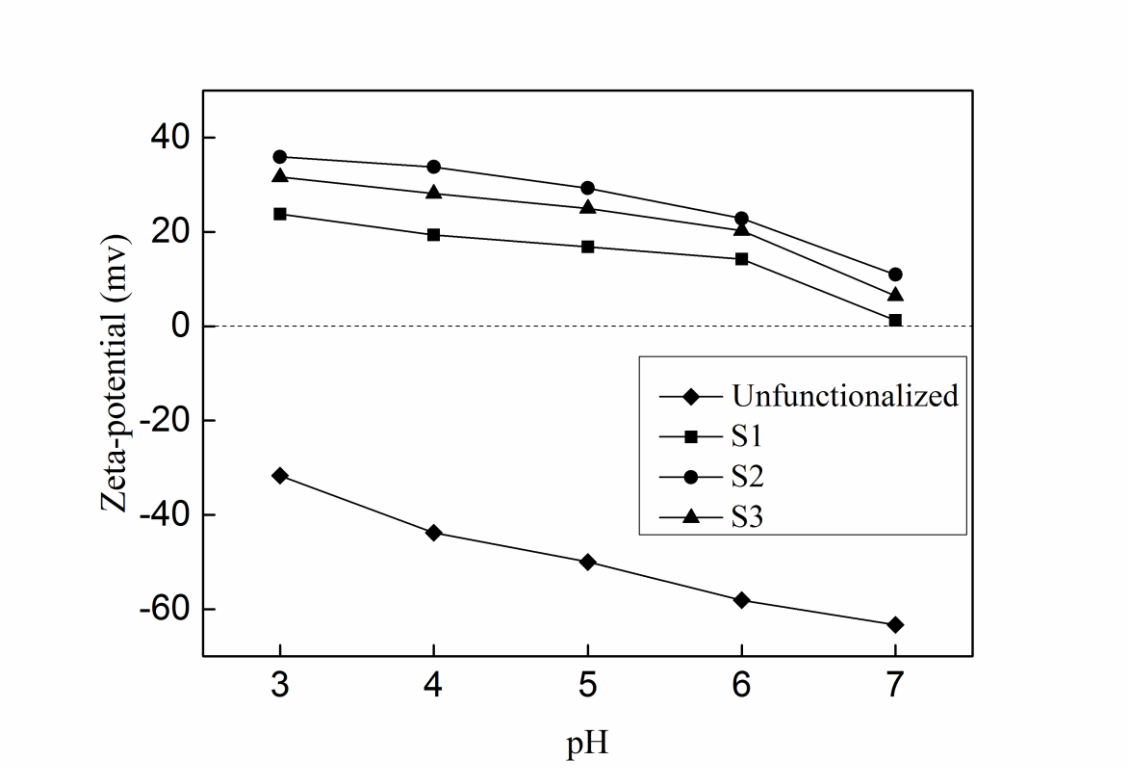


Fig. 4-2. Variation of zeta potential of three functionalized magnetic nanospheres and unfunctionalized magnetic nanospheres with pH

(c). Thermal analysis

In order to study the effect of silane structures on the grafting of silane on unfunctionalized magnetic nanospheres surface, TGA and DTA were carried out and the results are shown in Fig. 4-3. We can see that three functionalized magnetic nanospheres exhibited similar weight loss trend: the evaporation of adsorbed water and hydration water at about 145 °C; the organic silica start decomposing at about 200 °C; and the organic silane starts decomposing at about 400 °C (Fig. 4-3; S2; open).

However, each functionalized magnetic nanospheres weight loss is different at 30-600 °C, which is due to the different grafting percentage of organic silane onto Fe_3O_4/SiOx surface. S3 exhibits the largest weight loss of functionalized magnetic nanospheres, which consistents with the assumption in section 4.3.1.(c). Silane structure can influence the hydrolysis and grafting of the silane on unfunctionalized magnetic nanospheres surface. Owing to steric hindrance by alkyl spacer between the silane organo functionality and the silicon atom, S1 and S2 display lower weight loss as compared to S3. In addition, S2 shows more weight loss than that of S1 at 30-600 °C. This is probably attributed to the methoxy groups of S2 hydrolyzed more rapidly than the ethoxy groups of S1 [26, 37].

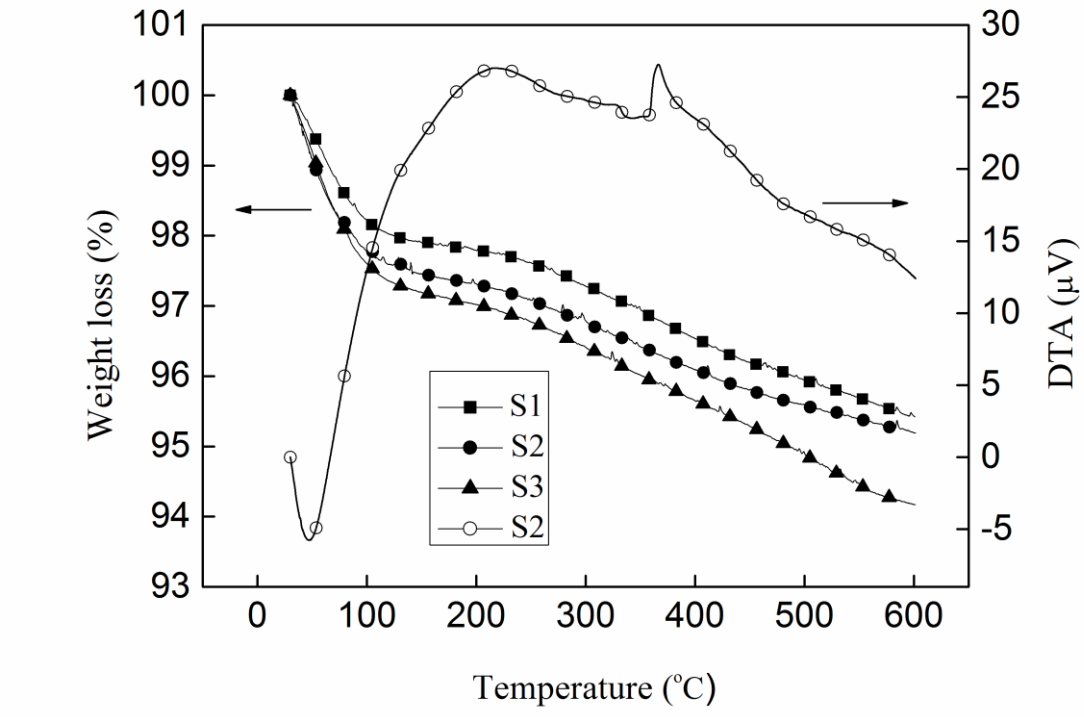


Fig. 4-3. Thermal analysis of three functionalized magnetic nanospheres: TGA of S1, S2 and S3 (solid); and DTA of S2 (open)

(d). Magnetite behavior

The magnetic properties of the three functionalized magnetic nanospheres were investigated by VSM at room temperature. Fig. 4-4 shows that the respective saturation magnetization of S1, S2 and S3 are 39, 37 and 32 emu/g, respectively, which is in

accordance with thermal analysis. Three functionalized magnetic nanospheres have large saturation magnetization, which can be separated from the reaction medium rapidly and easily for all further applications.

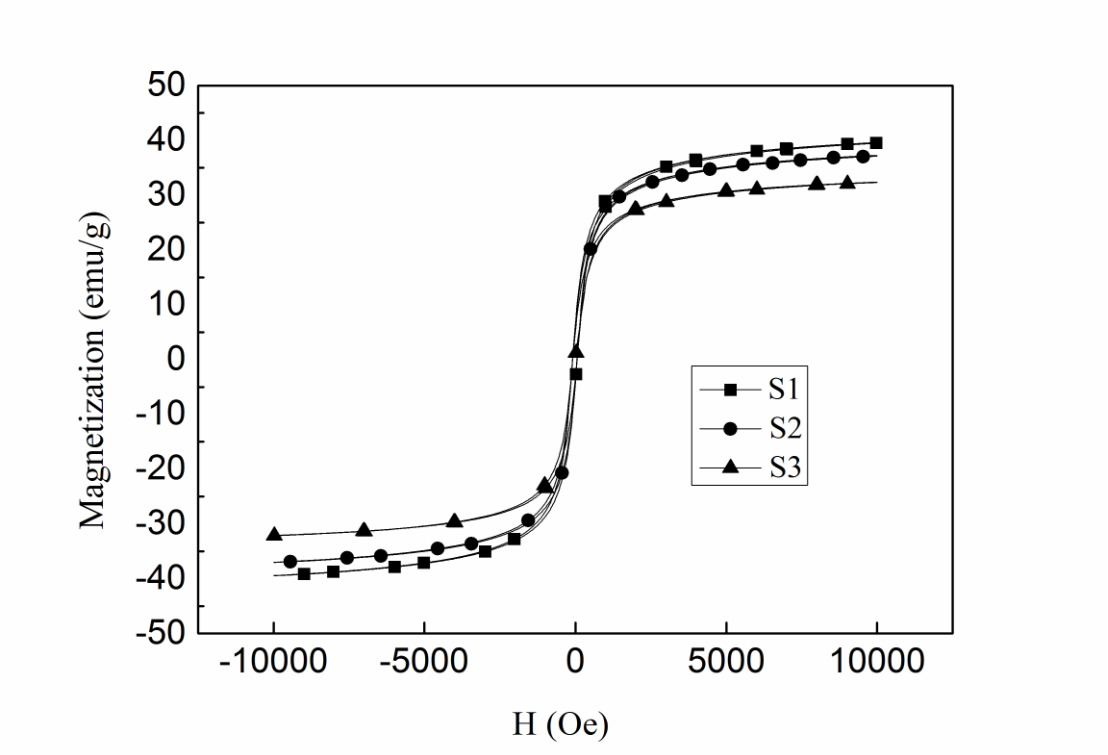


Fig. 4-4. Magnetite behavior of three functionalized magnetic nanospheres.

4.3.2. Optimum conditions for cellulase immobilization

In order to maximize the cellulase loading and the activity of the immobilized cellulase, the investigation of optimum conditions for cellulase immobilization were carried out.

(a). Effect of immobilization time

The effect of immobilization time on the amount of immobilized cellulase is shown in Fig. 4-5. The amount of immobilized cellulase greatly increases with reaction time from 0 to 6 h, and then levels off. Cellulase coverage is found to almost saturate within 6 h. However, Hirsh et al. [39] reported that the enzyme aggregate surface layer and enzyme conformation were found to change to orderly structure with further incubation time. It is considered that the competition between different enzymes in the mixture for physisorption sites and by the spreading of the adsorbed enzymes. Therefore,

immobilization of cellulase on functionalized magnetic nanospheres for incubation times up to 24 h.

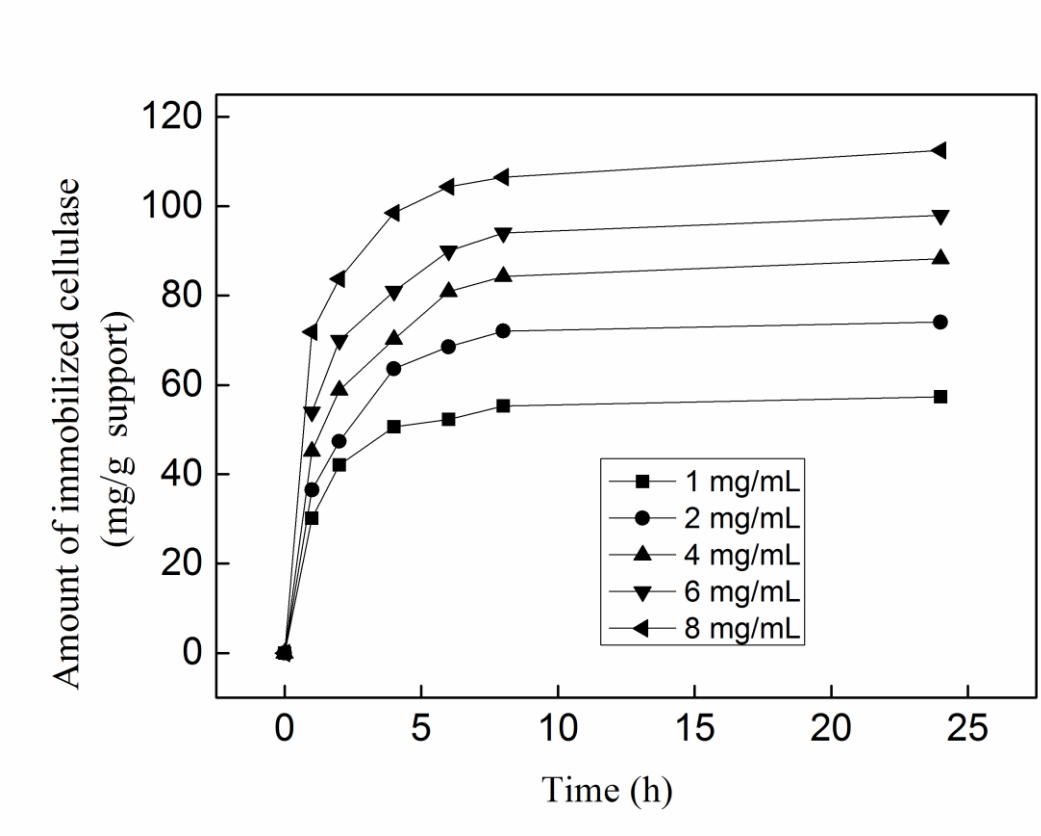


Fig. 4-5. Effect of immobilization time on the amount of immobilized cellulase of S2.

(b). Effect of initial cellulase concentration

The effect of initial cellulase concentration on the amount of the cellulase immobilized on functionalized magnetic nanospheres and loading efficiency of cellulase is shown in Fig. 4-6. The amount of immobilized cellulase increases greatly with the initial cellulase concentration in the solution from 0.0 to 4.0 mg/mL, then levels off. The loading efficiency of cellulase decreases with the increasing initial cellulase concentration. It is considered that the higher cellulase loading causes intermolecular steric hindrance by the cellulase, which restrains continuous immobilization of cellulase on the supports. Functionalized magnetic spheres exhibit higher cellulase adsorption capacity (89 mg/g supports) than the unfunctionalized magnetic spheres (Table 4-2).

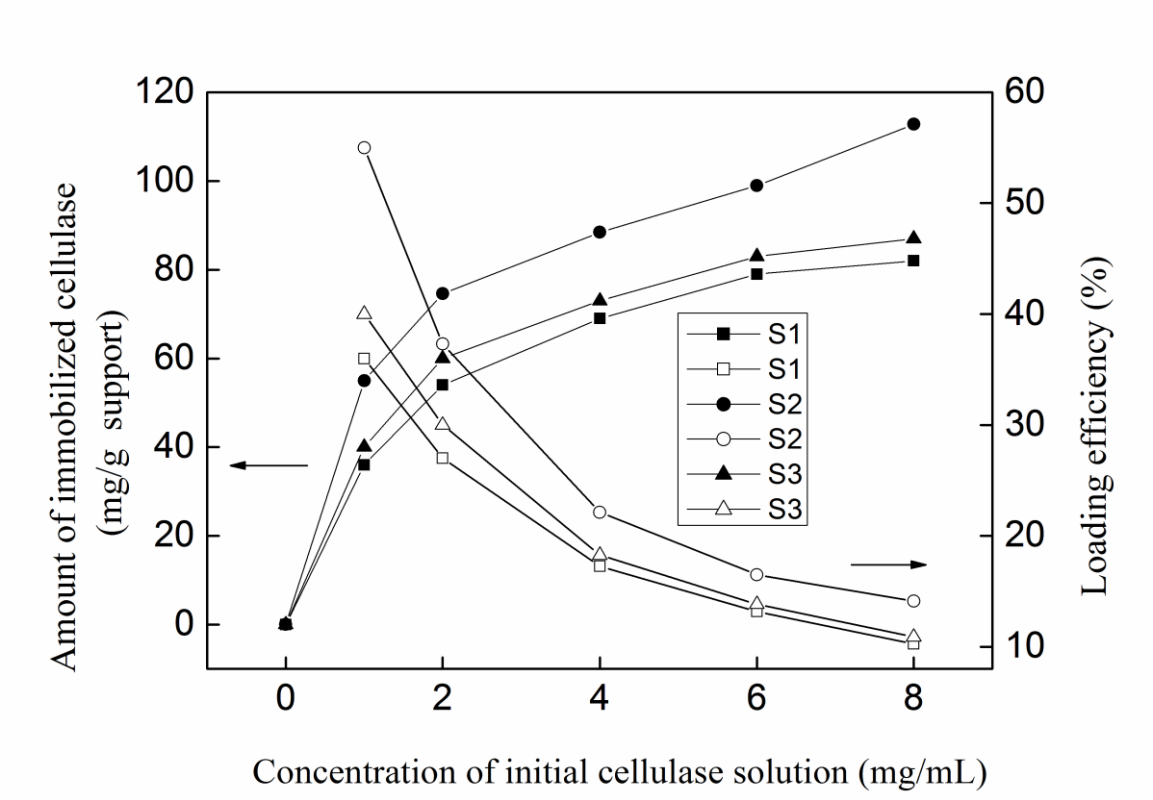


Fig. 4-6. Effect of concentration of initial cellulase on the amount of immobilized cellulase (solid) and loading efficiency (open)

Table 4-2. Cellulase loading capacity and zeta potential values of functionalized magnetic nanospheres and unfunctionalized magnetic nanospheres

Sample	Zeta potential at pH 5.0 (mV)	Amount of immobilized cellulase[a] (mg/g)	Activity of cellulase ($\mu mol \cdot mL^{-1} \cdot min^{-1}$)
unfunctionalized magnetic spheres	-40	62	0.29
S1	16	69	0.35
S2	29	89	0.42
S3	24	73	0.36

[a] 4 mg/mL concentration of initial cellulase

Silane modification have improved the capacity of cellulase immobilization, which is because immobilization of cellulase on functionalized magnetic nanospheres was

performed by electrostatic adsorption, and positive surface of functionalized magnetic nanospheres at pH 5.0 has a strong affinity with negatively charged cellulase [40]. S2 can adsorb comparatively more cellulase than the other functionalized magnetic nanospheres, which probably be attributed to highest a zeta potential of S2 (29 mV). This shows surface characteristics significantly influence the interaction of cellulase with supports surfaces. The activity recovery is 87% and the maximum amount of immobilized cellulase of S2 is 112 mg/g support at 8 mg/mL of initial cellulase.

4.3.3. Stability of immobilized cellulase

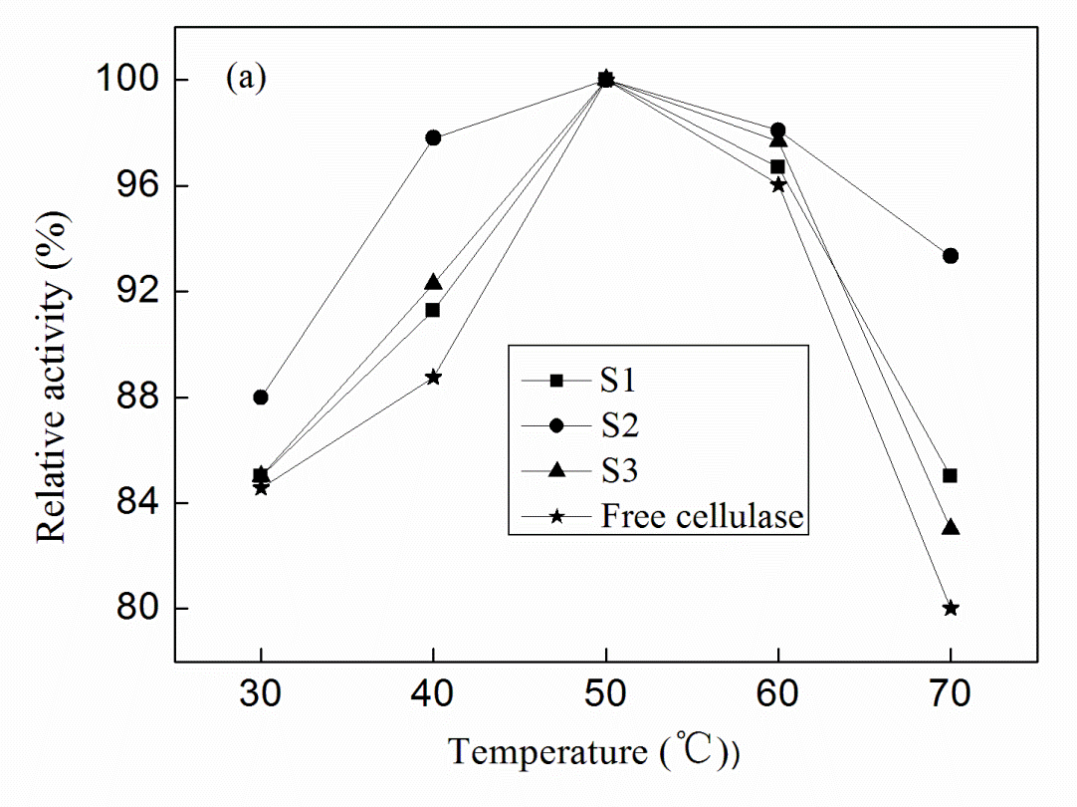
(a). pH and temperature

The effect of temperature of immobilized cellulase on hydrolytic activities of free and immobilized cellulase were determined and the results are shown in Fig. 4-7 (a). The optimum reaction temperature of free cellulase and the immobilized cellulase were both at 50 °C. The relative activity of immobilized cellulase was higher than that of the free cellulase, exhibiting a wider endurance for reaction temperature. Furthermore, the relative activity of S2 is about 97.8% at 40 °C, which holds better heat resistance at 40 °C than that of free cellulase and the other immobilization cellulases, demonstrating the potential of this immobilized cellulase for biofuel production in SSF process.

The optimum pH and pH stability of free and immobilized cellulase were determined within 3.0-7.0 pH range at 50 °C and results are shown in Fig. 4-7 (b). Both free cellulase and immobilized cellulase had a pH optimum at about 5.0. The immobilized cellulase has broader pH profiles than that of the free cellulase. Immobilized cellulase displayed a greater pH stability due to the carriers enhanced the cellulase rigidity.

(b). Thermal stability

Thermal stabilities of immobilized and free cellulase were compared by measuring their activities at 70 °C in acetate buffer solution pH 5.0. Fig. 4-8 demonstrates that immobilized and free cellulase exhibited a similar trend. However, immobilized cellulase has better thermal resistance than the free cellulase, and the similar phenomena were also observed in the previous reports [41]. The enhancement of thermal stability is probably attributed to the bonding between cellulase and this support, thus restricting the conformational change of cellulase during heating.



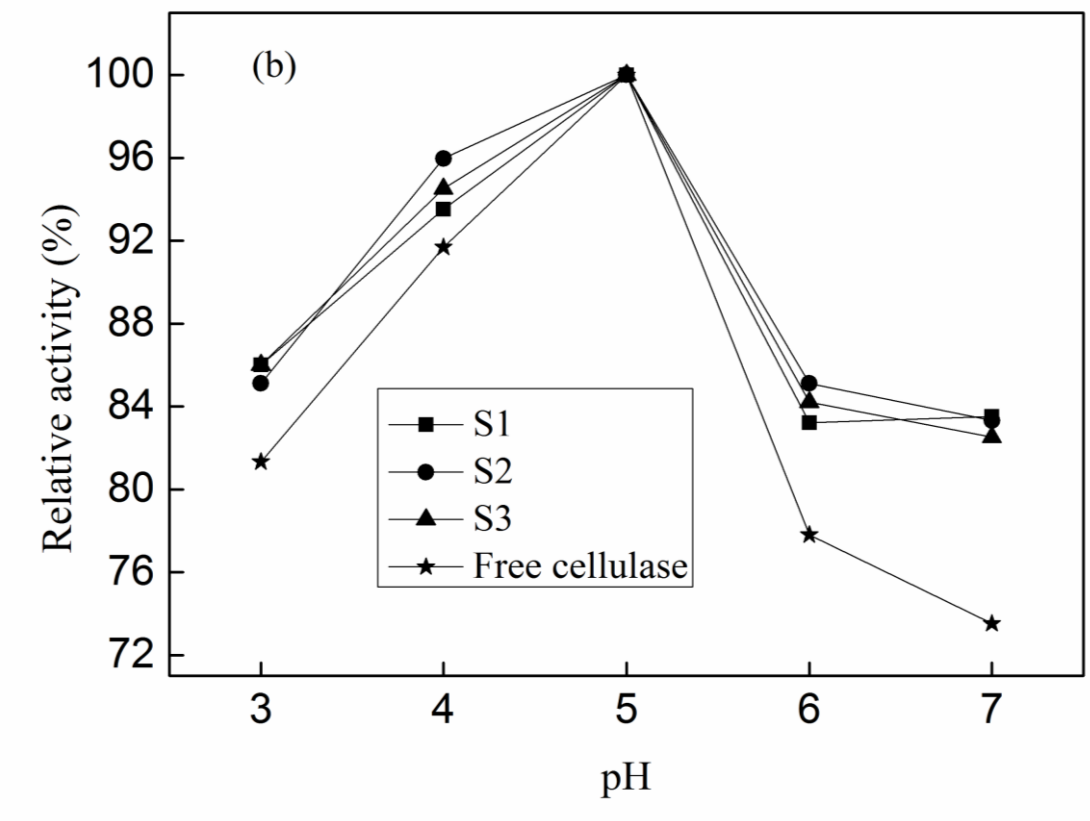


Fig. 4-7. Effect of temperature (a) and pH (b) on the relative activity and loading efficiency

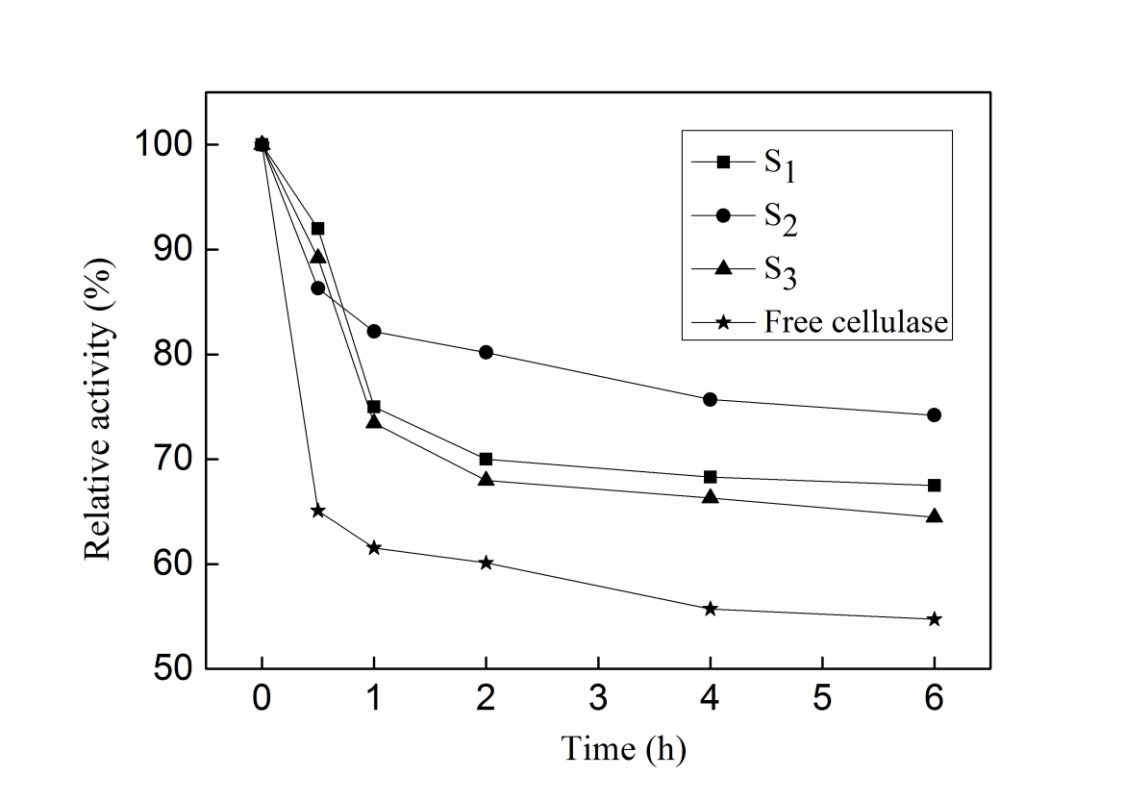


Fig. 4-8. Thermal stabilities of free and immobilized cellulase

4.3.4. Comparison of immobilization method

In order to investigate the influence of the immobilization method on cellulase immobilization, the properties of immobilized cellulase (S3) with (covalent binding; chapter 3) and without cross-linking agent (physical adsorption; chapter 4), such as the amount of immobilized cellulase, relative activity, stability and reusability were compared.

(a). Effect of concentration of initial cellulase solution on amount of immobilized cellulase and loading efficiency

The effects of initial cellulase concentration on the amount of the cellulase attached onto the support and on the relative activity are shown in Fig. 4-9. The amount of immobilized cellulase with and without cross-linking agent almost proportionally increases with the initial cellulase concentration in the solution from 0.0-4.0 mg/mL, then leveled off. The loading efficiency of cellulase decreased with the initial cellulase concentration. It is considered that the higher cellulase loading causes intermolecular steric hindrance by the cellulase, which restrains continuous immobilization of cellulase

on the supports. However, the amount of the immobilized cellulase with covalent cross-linking agent is clearly higher than that without cross-linking. The amount of immobilized cellulase without covalent cross-linking agent has a lower immobilization capacity. A similar result was reported by Hirsh et al. Immobilized cellulase without cross-linking agent depends on electrostatic interaction between support surfaces and cellulase. The structure of the adsorbed layer strongly depends on the competitive binding properties of the enzymes in the cellulase mixture.

The optimal condition of the initial cellulase concentration is 4.0 mg/mL. The amounts of immobilized cellulase with and without cross-linking agent are 92 and 73 mg/g support, respectively.

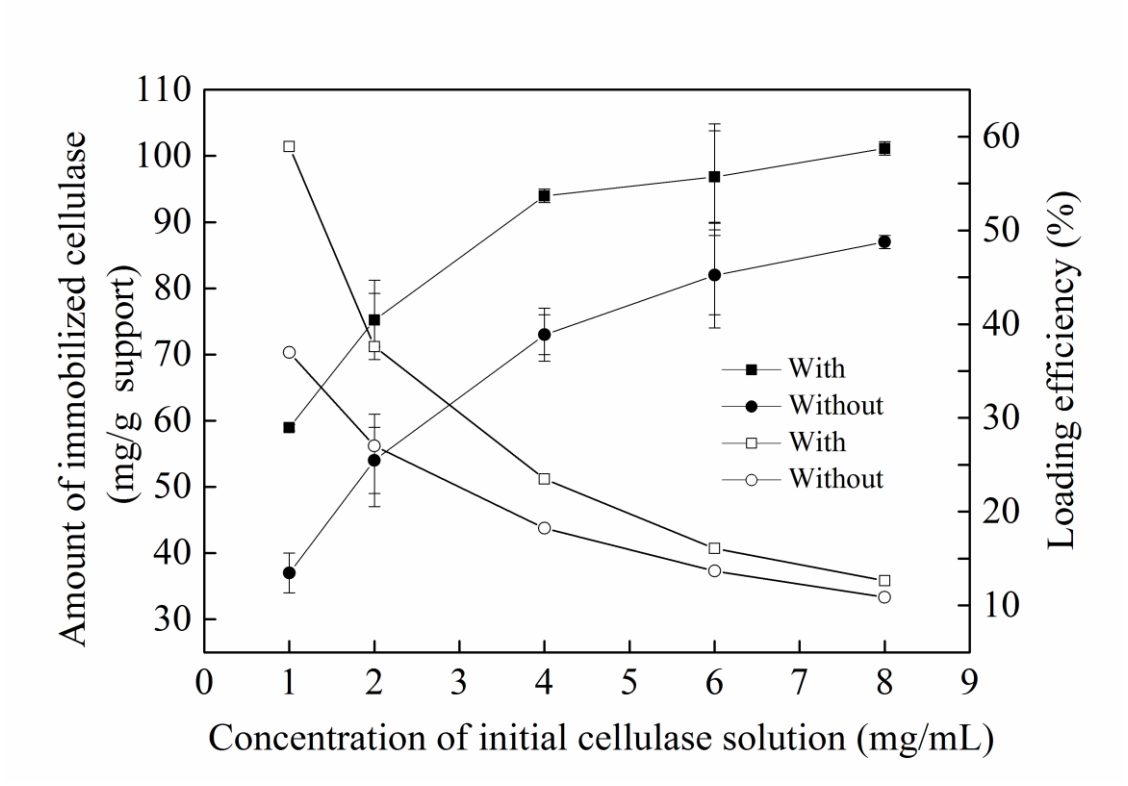


Fig. 4-9. Effects of immobilization method on the amount of immobilized cellulase and the loading efficiency

(b). Effect of concentration of initial cellulase solution on activity of cellulose

Fig. 4-10 shows that the relative activity of immobilized cellulase without cross-linking agent is higher than with the agent. Covalent attachment leads to partial inactivation of cellulase due to conformational restrictions caused by covalent binding

of the amino acid residues to the support, normally leading to improved enzyme stability. The respective activity recoveries of the immobilized cellulase with and without cross-linking agent are 80% and 90% when the initial cellulase concentration was 4.0 mg/mL. The results show that the immobilized cellulase with cross-linking agent provided better results as compared to other report.

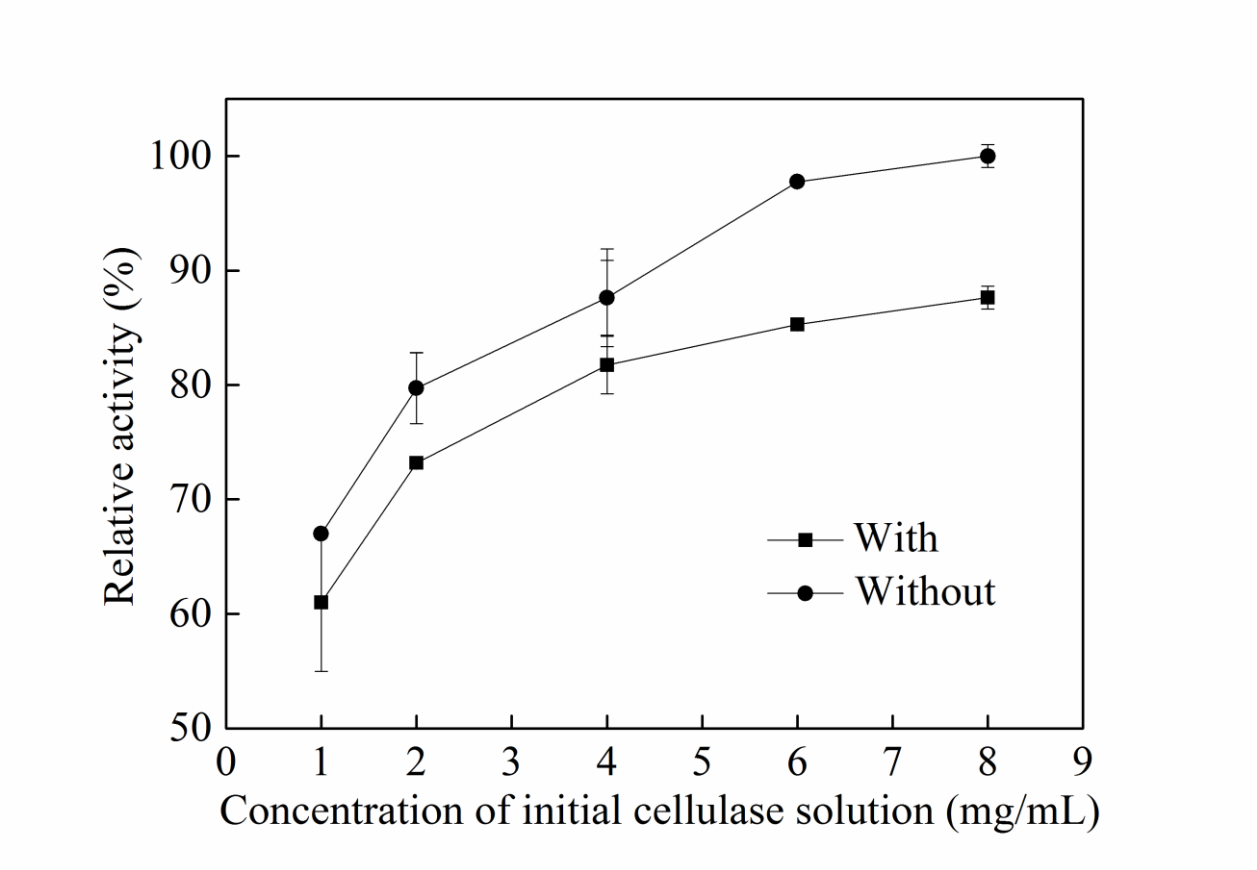


Fig. 4-10. Effect of immobilization method on activity of immobilized cellulase. CMC substrate was hydrolysis by immobilized cellulase at 50 °C.

(c). The reusability of immobilized cellulase

The reusability of immobilized cellulase is an important consideration in large-scale industrial application for economic reason. The reusability of immobilized cellulase with and without cross-linking agent was investigated and results as shown in Fig. 4-11. Immobilized cellulase with cross-linking agent has significant stability and residual activity is 85.5% of the initial activity after 10 continuous uses. By comparing our results with other reports. Cui et al. used amino-silane modified magnetic nanoparticles

to immobilize lipase, they retained only 58% of the enzyme activity by the ten use. The covalent immobilization cellulase on magnetic nanoparticles was studied by Jordan et al.. Immobilized cellulase retained only 10% of the enzyme activity by the 6 use. Thus immobilized cellulase with cross-linking agent has significantly high stability and good reusability for large-scale applications. However, the relative activity of immobilized cellulase without cross-linking agent is significantly decreased; the relative activity of immobilized cellulase decreases to 56% after 5 reaction cycles, and that is in accordance with other reports [32]. The decrease in activity likely come from the eventual cellulase leakage, that is desorption of a very small amount of residue cellulase absorbed on the supports; As explained in the previous section, cellulase shares a strong affinity for cellulosic substrates because of the presence of cellulose binding domains (CBDs). The forces responsible for physical adsorption of cellulase on supports are inherently weak and in the presence of cellulose a substrate with which cellulase enjoys natural chemistry, these interactions may not be strong enough to stop enzyme leaching.

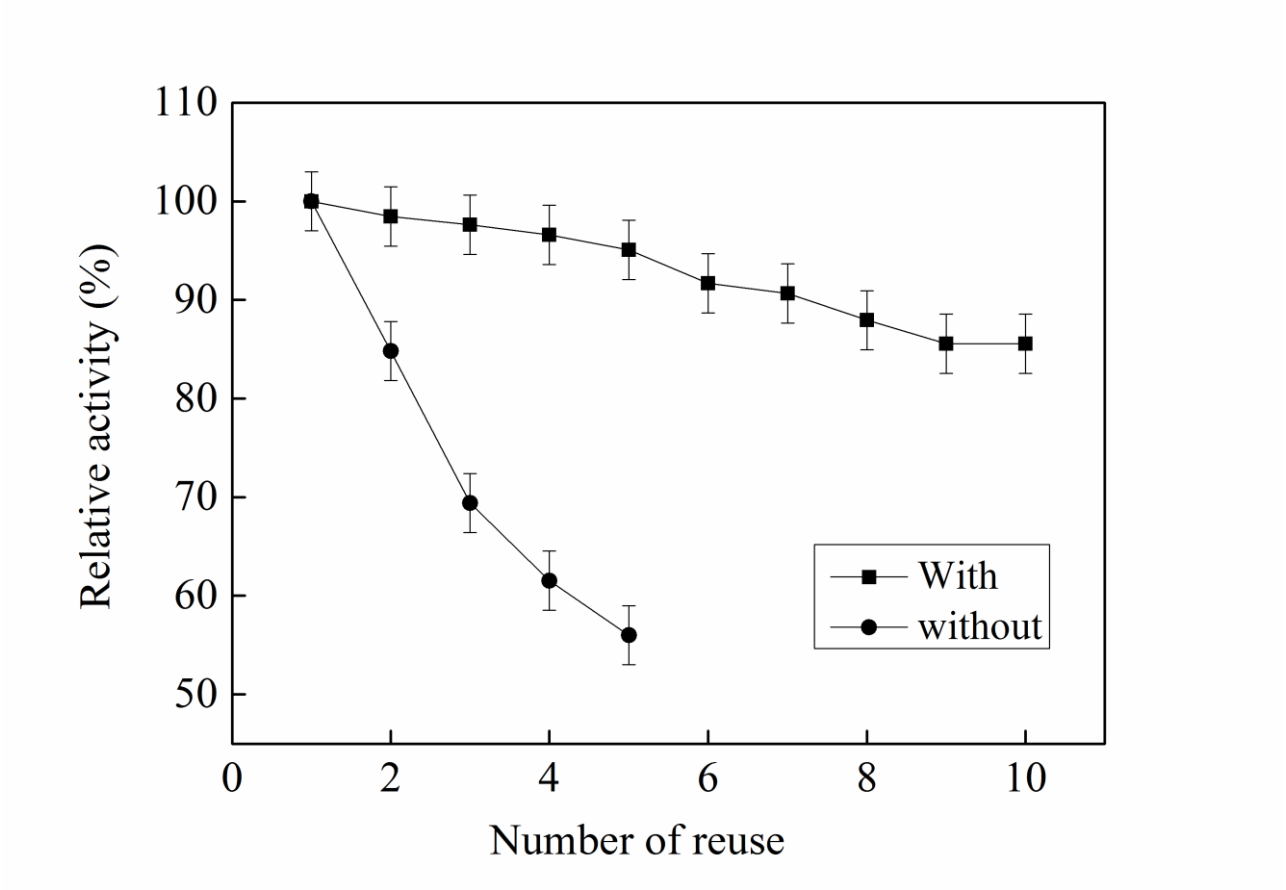


Fig. 4-11. Effect of immobilization method on reuse of immobilized cellulase. CMC substrate was hydrolysis by immobilized cellulase at 50 °C

The largest challenges in optimizing cellulosic ethanol production and hence increasing the feasibility of large-scale industrial applications are ensuring enzyme stability and reusability. Immobilized cellulase with cross-linking agent represents a significant approach to increase the efficiency of the enzymatic process that renders them reusable and hence reduces the enzyme-related operating costs.

4.4. Conclusion

The functionalized magnetic nanospheres were prepared by co-condensation of tetraethylorthosilicate with three different amino silanes: 3-(2-aminoethylaminopropyl) -triethoxysilane (AEAPTES), 3-(2-aminoethyl aminopropyl)-trimethoxysilane (AEAPTMES), and 3-aminopropyltriethoxysilane (APTES). The three functionalized magnetic nanospheres with core-shell morphologies exhibited improved dispersion. The saturation magnetization of S1, S2 and S3 are 39, 37 and 32 emu/g, respectively. AEAPTMES with methoxy groups was favored to be hydrolyzed and grafted on unfunctionalized magnetic nanospheres.

Cellulase was immobilized on three functionalized magnetic nanospheres and unfunctionalized magnetic nanospheres by electrostatic adsorption. The functionalized magnetic nanospheres exhibited higher the capacity of cellulase immobilization than unfunctionalized magnetic nanospheres. The increasing of surface charge of functionalized magnetic nanospheres leads to an enhancement of the capacity of cellulase immobilization. AEAPTMES modified functionalized magnetic nanospheres with highest a zeta potential (29 mV) exhibited 87% activity recovery and the maximum amount of immobilized cellulase of S2 is 112 mg/g support at 8 mg/mL of initial cellulase. Immobilized cellulase on functionalized magnetic nanospheres prepared by AEAPTMES exhibit higher temperature stability and broader pH stability, than other immobilized cellulase and free cellulase especially at 40 °C, demonstrating the potential of this immobilized cellulase for biofuel production in SSF process.

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Chapter 5 Preparation of magnetic composite microspheres by surfactant free controlled radical polymerization for cellulase immobilization

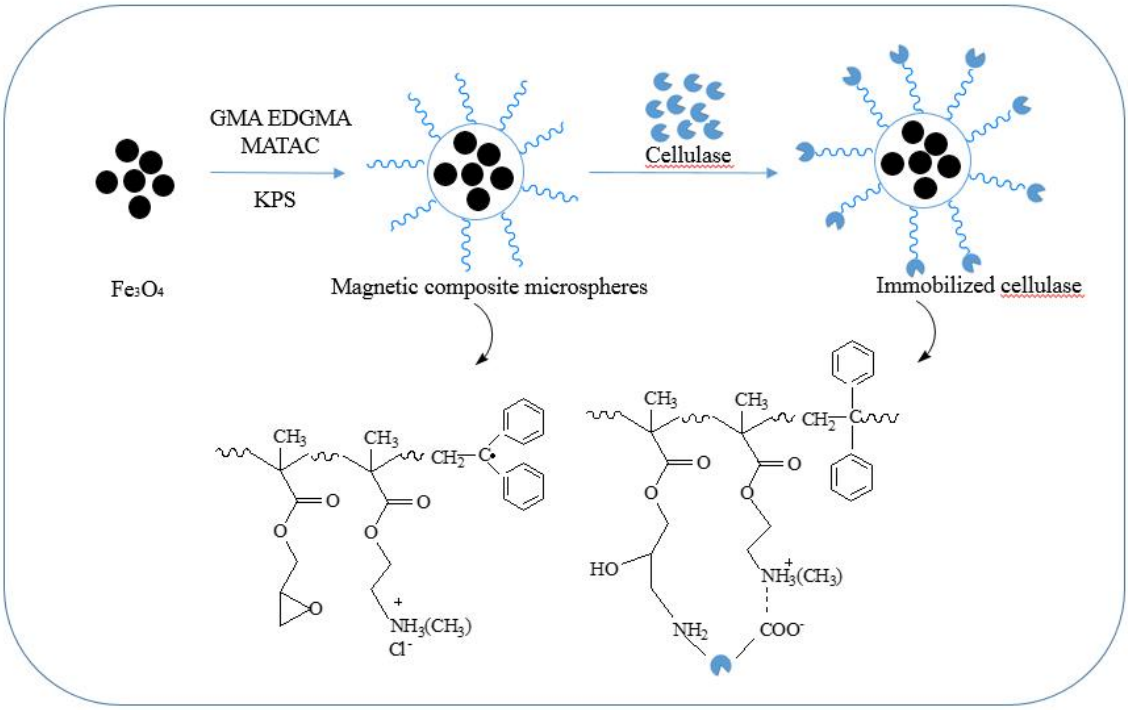
5.1. Introduction

Bioethanol produced from lignocellulosic biomass has been extensively studied in the past decades since the utilization of cellulosic biomass as a renewable resource has great potential for reducing emissions of carbon dioxide and thereby prevents global warming [1-3].

Cellulase is an enzymatic complex composed of endo-1,4-β-D-glucanases or endoglucanases, exo-1,4-β-D-glucanases or cellobiohydrolases and 1,4-β-D-glucosidases. Cellulase can effectively hydrolyze lignocellulosic biomass into fermentable sugar, which is subsequently converted into ethanol [4, 5]. Cellulase has some excellent properties (high activity, selectivity and specificity) that can allow hydrolysis processes to be carried out under the mild experimental and environmental conditions [6]. However, the use of cellulase is limited by the enzyme inactivation during the hydrolysis process and the difficulty to separate cellulase from the solution, which limits the further industrial applications of enzymatic hydrolysis of lignocellulosic biomass [7, 8]. One approach to increase the performance of enzymatic process is to immobilize the enzymes on solid supports which renders them reusable and hence reduces the enzyme-related operating costs [9, 10].

Recently, polymer grafted magnetic materials (magnetic composite microspheres) represent an attractive support family for use in enzyme technology [11, 12]. Living radical polymerizations are extensively used for the preparation of polymer grafted materials. It was found that the use of 1,1- diphenylethylene (DPE) in conventional free radical polymerization allows a high degree of polymer structural control. DPE is not a common monomer in the process of polymerization, and it is used as a chain transfer in radical polymerization. Controlled radical polymerization based on DPE has attracted some interest because it is commercially available, odorless, and without known toxicity

[13-15]. Magnetic composite microspheres consist of magnetic core and polymer shell have received much attention in enzyme immobilization [17-19]. Due to the existence of magnetic core, magnetic microspheres can be rapidly separated from the mixtures by a magnetic field. The polymer shell, which has some functional groups, can easily bind to many enzymes, protein, etc. In addition, magnetic composite microspheres has been increasingly used in immobilization of enzymes due to improved stability, the potential for modification of the catalytic properties, and in certain cases higher activity or selectivity.



Scheme 5-1. Synthesis of the magnetic composite microspheres and immobilization of cellulase

In this study, the novel magnetic composite microspheres were prepared by copolymerization of GMA and MATAC using DPE as radical control agent in the presence of Fe_3O_4 nanoparticles (Scheme 5-1). The structures and magnetic properties of magnetic composite microspheres were characterized by scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FT-IR) and thermogravimetric analysis (TGA). Magnetic composite microspheres were used as supports for immobilization of cellulase. The properties of immobilized cellulase were investigated, including the

amount of immobilized cellulase and reusability.

5.2. Experimental

5.2.1. Material

Meiji Acremonium cellulase (EN) was purchased from Meiji Seika Pharma Co., Ltd., Tokyo, Japan. Ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), ammonium hydroxide ($NH_3 \cdot H_2O$, 28 wt. %) and 1,1-diphenylethylene (DPE) were purchased from NacalaI Tesque, Inc, Kyoto, Japan.

5.2.2. Synthesis of magnetic composite microspheres

0.5 g of Fe_3O_4, certain amount of KPS, ethanol (44 mL) and distill water (10 mL) was put into a flask and vibrated with ultrasonic for 1 h. Then the flask was placed in a water bath at 50 °C under nitrogen atmosphere. A mixture of 4 mL GMA, 4 mL EGDMA, 5.1 mL MATAC, DPE, ethanol and H_2O were dropwise into the flask in 30 min. Then the reaction was carried out at 50 °C for 2 h, 60 °C for 2 h, 75 °C for 2 h. The magnetic composite microspheres were isolated from the solution by a magnet, and then washed several time with ethanol.

5.2.3. Immobilization of cellulase

Magnetic composite microspheres were put into 6 mg/mL cellulase solution (pH 5.0, acetate buffer solution) and were incubated at room temperature for 6 h. The resulting immobilized cellulase was washed several times with distilled water.

5.2.4. Reusability of immobilized cellulase

Reusability of immobilized cellulase was examined by hydrolysis of CMC by the recovered immobilized cellulase with magnetic separation and compared with the value of first run (activity defined as 100 %).

5.3. Results and discussion

5.3.1. Preparation and characterization of magnetic microsphere

(a). Morphologies

Micrographs of magnetic composite microspheres are shown in Fig. 5-1. The average

particle sizes of Fe_3O_4 nanoparticles ranged from 10 to 13 nm. After grafting of polymer, the average particle sizes of magnetic composite microspheres was about 2 um. The results indicate that the polymer chains had been effectively grafted onto the surface of Fe_3O_4 nanoparticles.

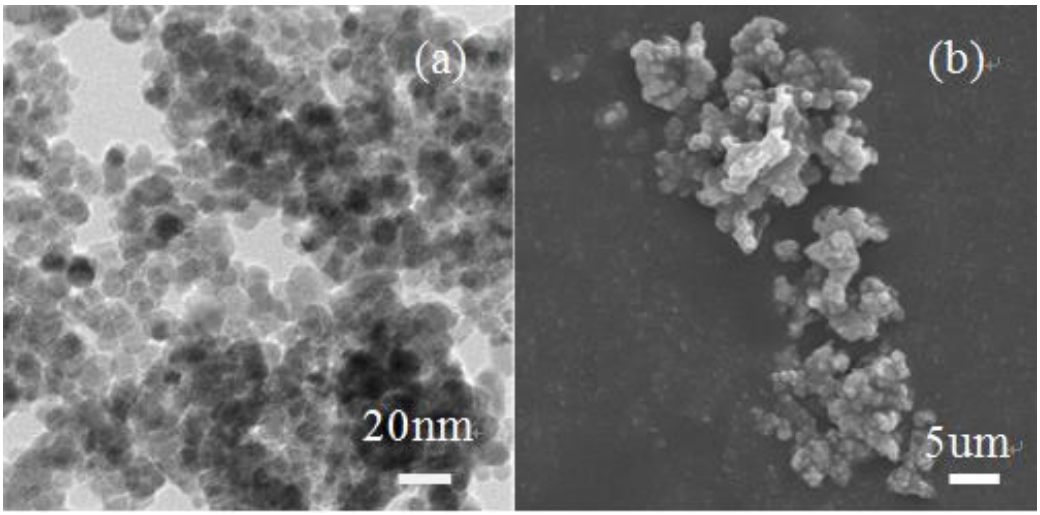


Fig. 5-1. (a) TEM image of Fe_3O_4, (b) SEM image of magnetic composite microspheres

(b). Binding confirmation

FT-IR analysis provides information regarding surface functional groups of magnetic poly (GMA-MATAC) and ensures immobilization of enzyme. FT-IR spectra of Fe_3O_4 (Fig. 5-2 (a)) shows the stretching vibrations of –OH at 3423 cm^{-1}, which is assigned to OH^- absorbed by Fe_3O_4 particles. The peak at 621 cm^{-1} is attributed to the Fe-O bond vibration of Fe_3O_4. FT-IR spectra of magnetic poly (GMA-MATAC) (Fig. 5-2 (b)) have the characteristic stretching vibration band of –OH of at around 3423 cm^{-1}. The vibration at 1719 cm^{-1} represents the ester configuration of both GMA and MATAC. Band at 1484, 1483 and 1381 cm^{-1} due to the bending vibration of $–CH_2$-, -CH- and $-CH_3$. In addition, the characteristic band at 1150 and 847 cm^{-1} belonged to the stretching vibration of the epoxy group from GMA. Two weak bands at 753 and 747 cm^{-1} can be assigned to monosubstituted aromatic groups, which may be due to the result of backbone vibrations of both phenyl rings. However, characteristic band of peak attributable to the stretching vibrations of C-N (normally at about 1100 cm^{-1}) was problematic due to overlapping other peaks.

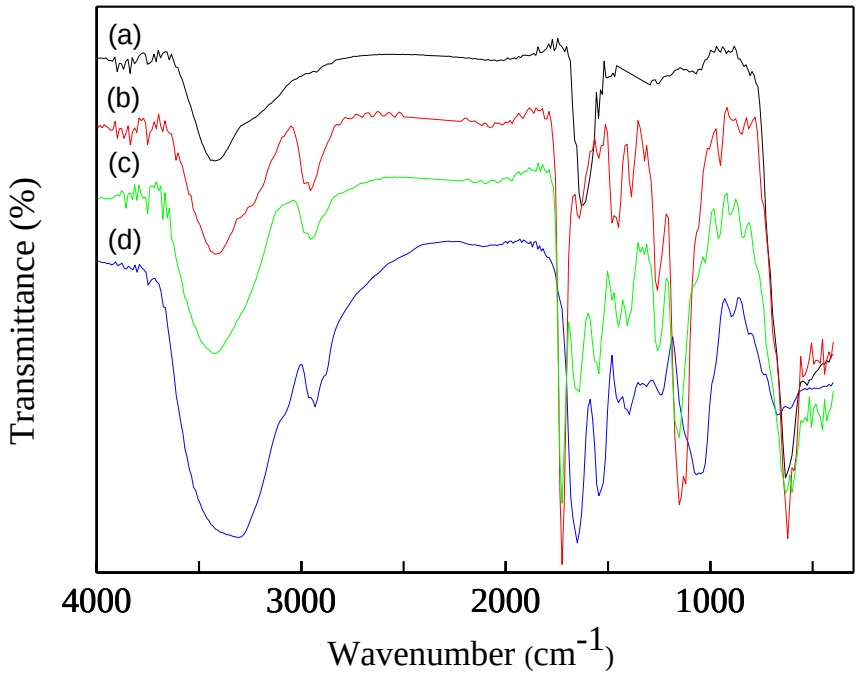


Fig. 5-2. FT-IR spectra of (a) Fe_3O_4, (b) Magnetic poly (GMA-MATAC), (c) Immobilized cellulase, (d) Cellulse

(c). Thermal analysis

In order to study grafting of polymer on Fe_3O_4 surface, TGA was carried out and the results are shown in Fig. 5-3. We can see that the magnetic composite microspheres start degrading at about 200 °C. The mass loss of magnetic composite microspheres is 40% from 100-600 °C. The mass loss of Fe_3O_4 is 1.86% from 100-600 °C. The result indicates that the magnetite content of the magnetic composite microspheres is 61%.

(d). Magnetic behavior

The magnetic properties of the magnetic poly (GMA-MATAC) were investigated by VSM analysis at room temperature. The mass magnetization curves of magnetic poly (GMA-MATAC) versus the applied magnetic field are presented in Fig. 5-4. The specific saturation magnetization was found to be for Fe_3O_4 and for magnetic poly (GMA-MATAC).The saturation magnetization is strongly dependent on the content of magnetite in the polymeric beads. The decrease in the saturation magnetization of beads after grafting with poly (GMA-MATAC) can be explained by the increase of the polymer content.

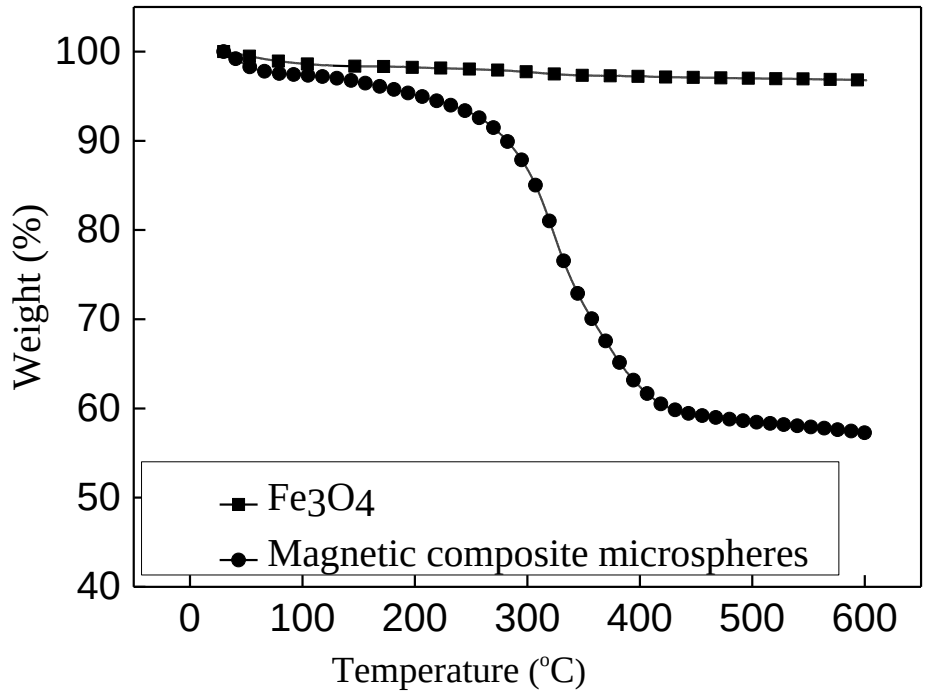


Fig. 5-3. TGA of Fe_3O_4 and magnetic composite microspheres

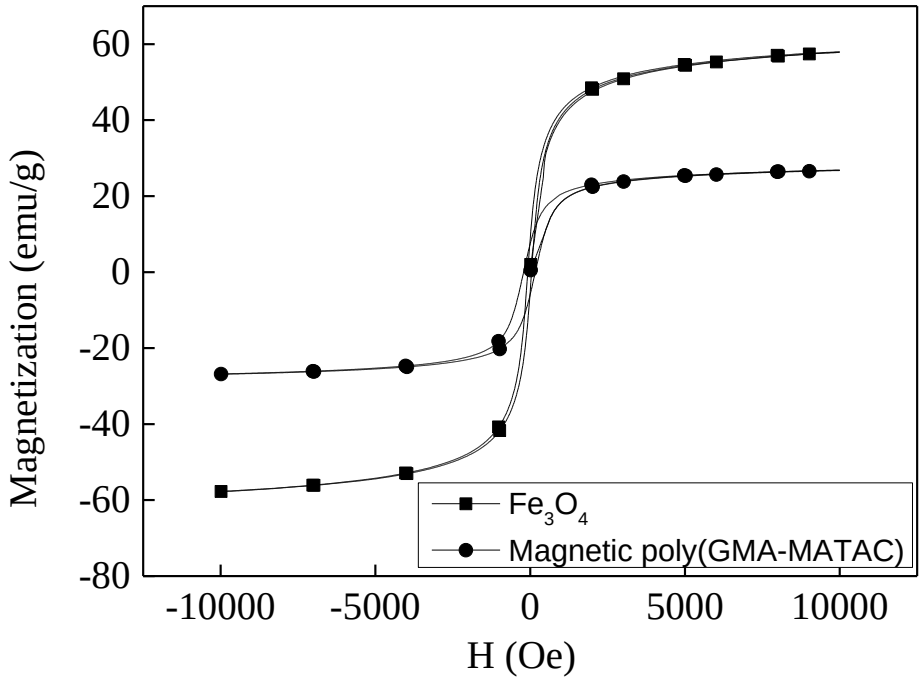


Fig. 5-4. Magnetic behaviors of (a) Fe_3O_4, (b) Magnetic poly (GMA-MATAC)

(e). Crystal structure

The crystal structure was investigated by XRD and Fig. 5-5 displays the XRD patterns of Fe_3O_4 and FMS. The Fe_3O_4 crystal with spinel structure has six diffraction peaks: {220}, {311}, {333}, {400}, {422} and {440}, and the present pattern peaks (curve (a)) are in agreement with them. The size of Fe_3O_4 can be calculated according to the Scherrer equation, and their average crystalline size of the magnetic particles is about 11.9 nm. These peaks were observed for magnetic poly (GMA-MATAC) (curve (b)). This means that coating and modifying on the surface of Fe_3O_4 does not lead to any change of the spinel structure.

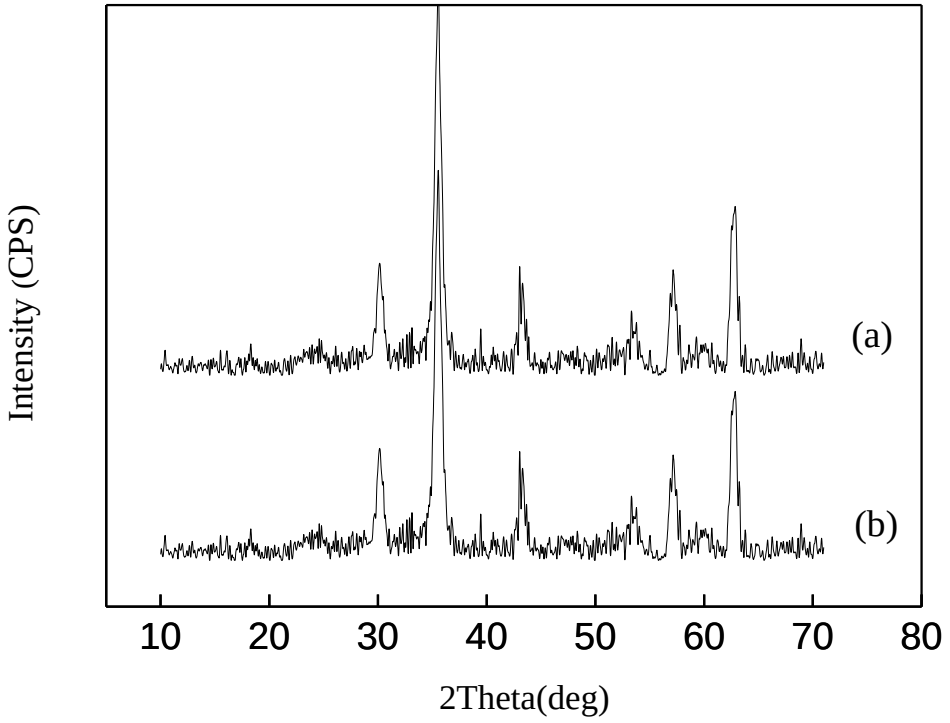


Fig. 5-5. Wide-angle X-ray diffraction patterns of (a) Fe_3O_4, (b) magnetic poly (GMA-MATAC)

5.3.2. The immobilization capacity of magnetic composite microspheres

The effects of initial cellulase concentration on the amount of the cellulase attached onto the support are shown in Fig. 5-6. The amount of immobilized cellulase almost increases with the initial cellulase concentration in the solution from 0.0-6.0 mg/mL,

then leveled off. The amounts of immobilized cellulase are 153 mg/g support at 6 mg/mL concentration of initial cellulase. The results show that the immobilization cellulase on magnetic composite microspheres exhibited higher immobilization capacity as compared to other report, which is because the pores formed during the polymerization procedure and pores increase in the surface area.

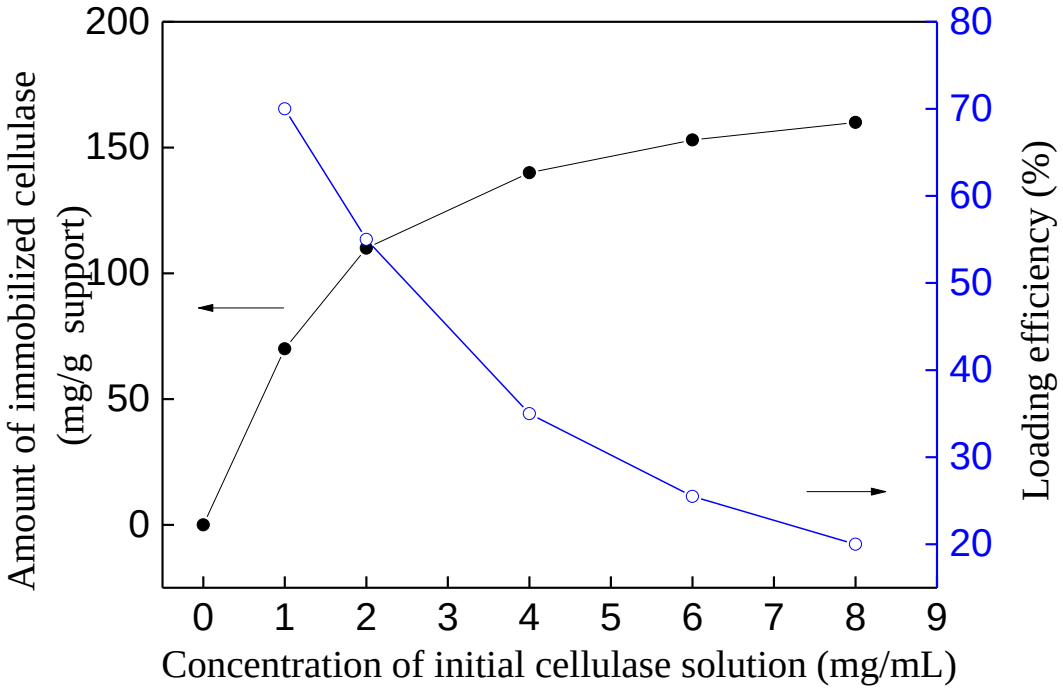


Fig. 5-6. Effects of concentration of initial cellulase solution on the amount of immobilized cellulase and the loading efficiency

5.3.3. Optimum conditions of enzymatic activity

The effect of pH on the activity of free and immobilized cellulase was carried out in the pH range 3-7 (Figure 5-7 (a)). As seen in this figure, optimal conversion was observed at pH 5 for the free enzyme, whereas the optimum pH value was slightly shifted to the acidic region upon immobilization. The immobilized cellulase has a different pH activity profile compared to free enzyme and was broadened in both alkaline and acidic regions. The temperature dependence of the activity of the free and immobilized cellulase was studied in the temperature range of 30-70 °C (Figure5-7 (b)).

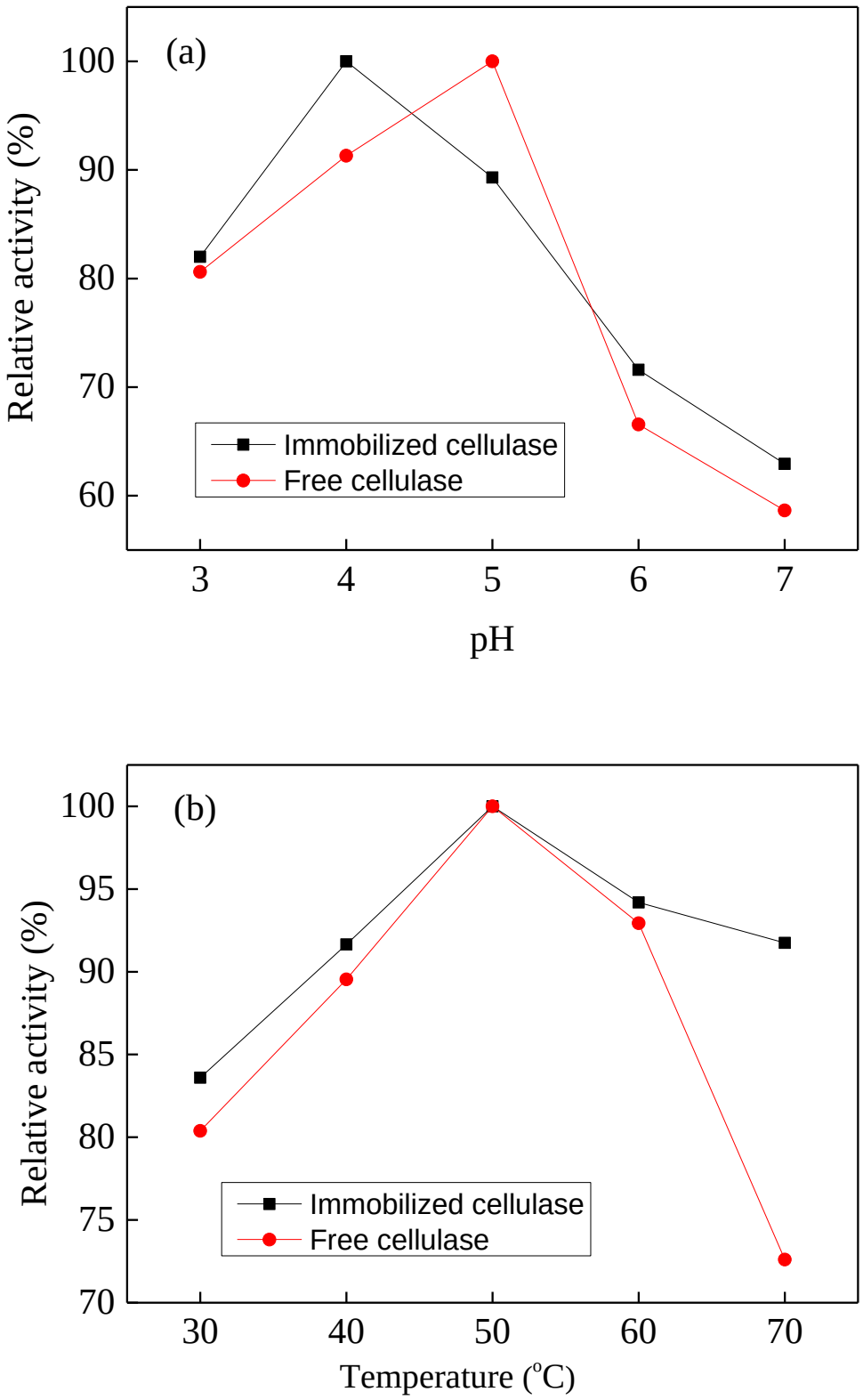


Fig. 5-7. Effects of pH and temperature on the activity of free cellulase and immobilized cellulase: (a) pH and (b) temperature

The optimum reaction temperature of free cellulase and the immobilized cellulase were both at 50 °C. Immobilized cellulase yielded higher activity over a wider range of temperatures than did the free cellulase. The change in pH and temperature for the immobilized cellulase could be due to the change in physical and chemical properties during the modification process. The applied immobilization protocol and covalent binding might also reduce the flexibility of cellulase molecule and result in an increase in the activation energy of the enzyme to bind its substrate.

5.3.4. Reusability of immobilized cellulase

The reusability of immobilized cellulase is an important consideration in large-scale industrial application for economic reason. The reusability of immobilized cellulase was investigated and results as shown in Fig. 5-8.

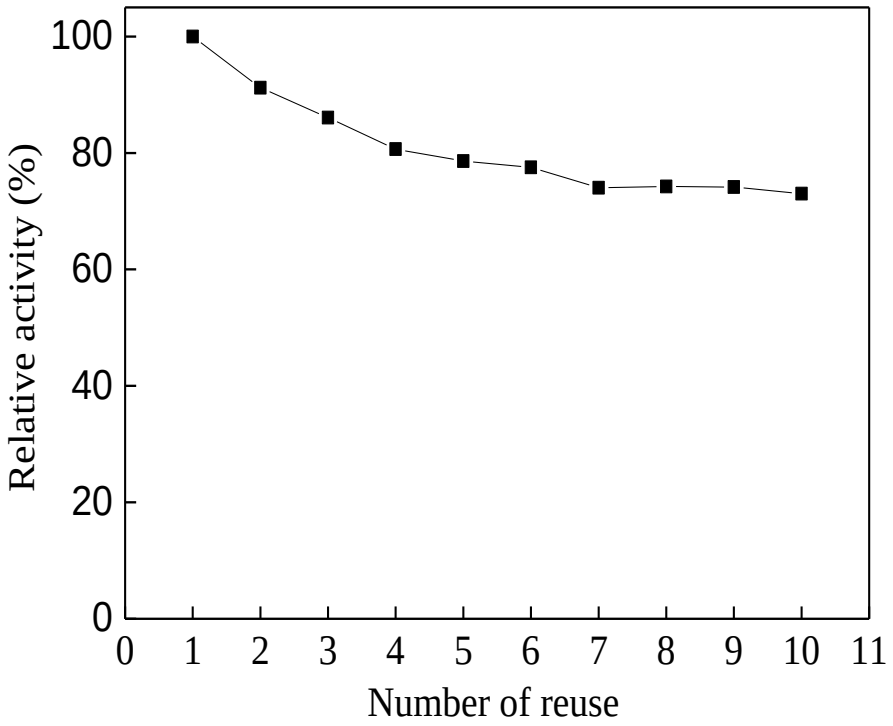


Fig. 5-8. The reuse of immobilized cellulase

The activity of immobilized cellulase significantly decreased from reaction cycles from 1 to 5, the activity becomes stable after that. The decrease in activity likely come

from the eventual cellulase leakage, that is desorption of a very small amount of residue cellulase absorbed on the supports; but the immobilized cellulase does not become completely inactivated after 10 reaction cycles. Immobilized cellulase has significant stability and retains 72% of initial activity after 10 continuous uses.

5.4. Conclusion

The novel magnetic composite microspheres were prepared by copolymerization of glycidyl methacrylate and methacryloxyethyl trimethyl ammonium chloride using DPE as radical control agent in the presence of Fe_3O_4 nanoparticles. The structures and magnetic properties of magnetic composite microspheres were characterized. The results indicate that the polymer chains had been effectively grafted onto the surface of Fe_3O_4 nanoparticles. The average particle sizes of magnetic composite microspheres was about 2 um. Cellulase was immobilized on magnetic composite microspheres by electrostatic adsorption and covalent binding with 153 mg/g supports. The relative activity of the immobilized cellulase was still 72% of the initial activity after 10 continuous uses.

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Chapter 6 Conclusions

6.1. General conclusions and remarks

Magnetic particles are the most often used supports because of their good biocompatibility, magnetic susceptibility, low toxicity, and ease of preparation in the desired size. Magnetic particles are important material for a wide range of application, such as bioseparation, biocatalysis, virus detection and biomolecules immobilization, which was due to the ease of separating biomolecules from reaction solutions, their recyclability and preventing of biomolecules dissociation. In the present study, several kinds of magnetic particles are investigated, then those magnetic particles were used as supports for immobilization of cellulase. Immobilized cellulase on magnetic supports had higher temperature stability and broader pH stability than free cellulase, demonstrating the potential of this immobilized cellulase for large-scale biofuel production.

In chapter 1, the research backgrounds, research significance, summary of the research and the construction of this thesis are described. The objectives of the research are to study synthesis magnetic supports and strategy of immobilized enzyme.

In chapter 2, the properties of magnetic supports and immobilized enzyme are presented. The experimental methods and characteristics are also presented in this chapter.

In chapter 3, functionalized magnetic silica nanospheres were prepared in two steps: by silica coating magnetite nanoparticles and then making an amino-silane modification of the silica-coated magnetite particles. The effects of different amounts of tetraethylorthosilicate on the morphologies and magnetic properties of the silica-coated magnetite particles were investigated and the structures and properties of the functionalized magnetic silica nanospheres were characterized. When the Fe_3O_4 nanoparticles were reacted with 5 mL of TEOS as the optimal amount, the MS_5 prepared with core-shell structure had 37% silica content and good dispersion. Amino silane was coupled to the surface of silica-coated magnetite nanoparticles, which was proved by FT-IR and DTA. Wide-angle X-ray diffraction pattern of FMS nanospheres showed that they had the spinel structure similar to Fe_3O_4. The FMS nanospheres prepared with 5 mL of TEOS was used as support for immobilization of cellulase. The

amount of immobilized cellulase was 92 mg/g support and the activity recovery was 80%. Immobilized cellulase exhibited better resistance to high temperature in comparison to free cellulase. The activity of the immobilized cellulase is still 63.2% retention of the initial activity after 10 continuous uses, which demonstrated the large-scale industrial applications.

In chapter 4, the functionalized magnetic nanospheres were prepared by co-condensation of tetraethylorthosilicate with three different amino silanes: 3-(2-aminoethylaminopropyl) -triethoxysilane (AEAPTES), 3-(2-aminoethyl aminopropyl)-trimethoxysilane (AEAPTMES), and 3-aminopropyltriethoxysilane (APTES). AEAPTMES with methoxy groups was favored to be hydrolyzed and grafted on unfunctionalized magnetic nanospheres. Cellulase was immobilized on three functionalized magnetic nanospheres and unfunctionalized magnetic nanospheres by electrostatic adsorption. The functionalized magnetic nanospheres exhibited higher the capacity of cellulase immobilization than unfunctionalized magnetic nanospheres. The increasing of surface charge of functionalized magnetic nanospheres leads to an enhancement of the capacity of cellulase immobilization. AEAPTMES modified functionalized magnetic nanospheres with highest a zeta potential (29 mV) exhibited 87% activity recovery and the maximum amount of immobilized cellulase of S2 is 112 mg/g support at 8 mg/mL of initial cellulase. Immobilized cellulase on functionalized magnetic nanospheres prepared by AEAPTMES exhibit higher temperature stability and broader pH stability, than other immobilized cellulase and free cellulase especially at 40 °C, demonstrating the potential of this immobilized cellulase for biofuel production in SSF process.

In chapter 5, the novel magnetic composite microspheres were prepared by copolymerization of glycidyl methacrylate and methacryloxyethyl trimethyl ammonium chloride using DPE as radical control agent in the presence of Fe_3O_4 nanoparticles. The structures and magnetic properties of magnetic composite microspheres were characterized. The results indicate that the polymer chains had been effectively grafted onto the surface of Fe_3O_4 nanoparticles. The average particle sizes of magnetic composite microspheres was about 2 um. Cellulase was immobilized on magnetic composite microspheres by electrostatic adsorption and covalent binding with 153 mg/g supports. The relative activity of the immobilized cellulase was still 72% of the initial activity after 10 continuous uses.

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

immobilized enzyme on the different supports was compared under the same experiment condition. The next works to be carried out are also prospected.

Table 6-1 Comparison of the performance of the immobilized enzyme on the different supports

Magnetic supports	**Immobilization strategy**	**Amount of immobilized cellulase**	**Activity recovery**	**Reusability**
FMS (chapter 3)	Covalent binding	92 mg/g	80 %	85.5 %
S3 (chapter 4)	Adsorption	73 mg/g	90 %	58 %
S2 (chapter 4)	Adsorption	89 mg/g	87%	64 %
Magnetic composite microspheres (chapter 5)	Covalent binding and adsorption	153 mg/g	73%	72 %

* FMS (chapter 3) is same as S3(chapter 4)

6.2. Future work

In recent years, bioethanol is one of the most promising biofuels to lessen the environmental pollution generated by burning of fossil fuels. Immobilization of cellulase on magnetic supports has been increasingly used to convert lignocellulosic into bioethanol. Magnetic particles were used as supports for immobilization cellulase due to reusability, biocompatibility, and improved stability. Magnetic particles have been studied for several decades, lots of technology have been practical applied. However, magnetic particles used as supports for immobilization of enzyme exhibit low immobilization efficiency, activity, and reusability and so on. Efficient strategy should

be conducted to solve these problems.

(1) Magnetic particles biodistribution appears to be significantly influenced by its physicochemical properties. The immobilization of enzymes onto nano-scale materials provides a reduction in the size of the enzyme-support materials for the attachment of enzymes, higher enzyme loading per unit mass of particles. However, nano-scale Fe_3O_4 particles tend to aggregate to form clusters. Design the nano-scale magnetic particles to improve immobilization efficiency and prevent nanparticles aggregation.

(2) Entrapment enzyme on/in mesoporous materials is attributed to its high surface area, uniform pore distribution, tunable pore size and high adsorption capacity. Prepare magnetic mesoporous materials to improve immobilization efficiency.

(3) Synthesis the novel magnetic composite microspheres with multifunctional group to improve immobilization efficiency, activity, and reusability and so on.

Publications

Ⅰ．審査付投稿論文

[1] **Wenjuan Zhang**, Jianhui Qiu, Huixia Feng, Xueli Wu, Limin Zang, Eiichi Sakai. Synthesis and characterization of functionalized magnetic silica nanospheres for the Acremonium cellulase immobilization. Journal of Nanoscience and Nanotechnology, In Press, (IF=1.339)

[2] **Wenjuan Zhang**, Jianhui Qiu, Limin Zang and Eiichi Sakai. Preparation of functionalized magnetic silica nanospheres for the cellulase immobilization. Nano, 10 (2015) 1550013, (IF=1.26)

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注：博士論文テーマ関連：4 編（(1）～（4)），その他：2 編（(5）～（6)）

Ⅱ．国際会議論文・発表

[1] **Wenjuan Zhang**, Jianhui Qiu, Huixia Feng, Eiichi Sakai. Immobilization of cellulase on functionalized magnetic nanospheres and determination of its activity and stability. The 59th Annual Magnetism and Magnetic Materials (MMM) Conference. Honolulu, Hawaii, November 3-7, 2014.

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注：博士論文テーマ関連：2 件

Ⅲ. 国内学会

[1] **張　文娟**、邱　建輝、境　英一. 磁性複合微粒子によるセルラーゼの固定化. 2014 年 9 月 第 39 回複合材料シンポジウム, 秋田市, 講演論文集, No. A1-06, pp. 17.

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