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Lipase immobilization on amino-silane modified superparamagnetic Fe_3O_4 nanoparticles as biocatalyst for biodiesel production

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ABSTRACT

In this work, superparamagnetic Fe_3O_4 nanoparticles were synthesized by chemical co-precipitation using ionic liquid of 1-Butyl-3-methylimidazolium tetrafluoroborate ([BMIN]BF_4) as templates. Then, the magnetic Fe_3O_4 nanoparticles were treated with 3-aminopropyltriethoxysilane (APTES) and obtain the surface amino-functionalized magnetic nanoparticles (APTES-Fe_3O_4) were used as immobilization material. Lipase was covalently bound to the APTES-Fe_3O_4 magnetic nanoparticles by using glutaraldehyde as a coupling reagent. A fatty acid methyl ester (FAME) conversion of 89.4% could be achieved by lipase immobilized on APTES-Fe_3O_4 magnetic nanoparticles under optimized conditions of methanol-oil molar ratio of 6:1, with a catalyst dose of 20%, 2% water content and 250 rpm agitation speed at 45 °C for 24 h. The FAME conversion remained greater than 70% even after reusing the catalyst for 5 reactions. In addition, the lipases immobilized on APTES-Fe_3O_4 magnetic nanoparticles could be recovered easily by external magnetic field for further use.

1. Introduction

Biodiesel has attracted considerable interest as sustainable fuels because of its low sulfur content, low hydrocarbon aroma, high cetane number, high flash point, and low environmental impact [1,2]. Biodiesel is a mixture of long-chain fatty acid methyl esters (FAME) produced from the transesterification of triglycerides, derived from vegetable oils or animal fats, with methanol [3–5]. Typically, the commercial production of biodiesel involves the use of strongly acid or strongly basic solutions (i.e. NaOH, KOH, and H_2SO_4) as catalysts [6–8] However, these catalysts have several drawbacks: they are corrosive, cause saponification of fatty acids, and produce high quantities of waste, leading to the release of environment-unfriendly effluents [9,10].

Enzymatic conversion of biodiesel has been suggested as a realistic alternative to the conventional methods [11,12]. Enzymatic production of biodiesel have several advantages including purer products, easy separation of glycerol byproduct, higher compatibility with feed stocks containing higher levels of free fatty acids, and ability to operate at ambient conditions [13,14]. However, the production of biodiesel by enzymatic method has not been adopted industrially, because of the high cost of enzyme catalyst and inconvenience in their separation, recycling and reusing. In order to use the enzyme catalyst repeatedly, the use of immobilized lipase onto insoluble or solid support is a possible solution to this problem [15–18].

Magnetic Fe_3O_4 nanoparticles as a new type of material [21] used as the carrier of immobilized lipases, have unique properties including the smaller size of the core [19], the larger the surface area for functionalization [20], the higher coercive force, the stronger surface adsorption ability [21], the better suspension property [22], superparamagnetic behavior and low toxicity [23], easy separation and effective recycle under external magnetic field, etc. Moreover functionalized magnetic Fe_3O_4 nanoparticles may facilitate covalent bonding of enzymes making it possible for site specific immobilization which adds to the better stability [24], enzyme activity and reusability of enzymes [25]. The functionalized magnetic nanoparticles have been used by many researchers to immobilize lipase for various applications due to its facile synthesis, chemical stability, nontoxic and non-carcinogenic nature [26–33].

In this research, superparamagnetic Fe_3O_4 nanoparticles were prepared *via* ionic liquid [BMIN]BF_4 assisted co-precipitation. Then amino-silane modified Fe_3O_4 nanoparticles were prepared and used as the

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support for immobilization of lipase. The immobilization was confirmed by FT-IR, TEM, XRD and VSM techniques. Finally, the application of the immobilized lipase in synthesis of biodiesel was also estimated. The effects of various reaction parameters that might influence the yield of FAMEs, including water content, reaction time, molar ratio of substrates, and catalyst dosage, were explored in this experiment.

2. Experimental

2.1. Materials and reagents

APTES (3-aminopropyltriethoxysilane) was purchased from Sigma-Aldrich. [BMIN]BF_4 (1-Butyl-3-methylimidazolium tetrafluoroborate) was purchased from the Shanghai Chengjie chemical company. Rapeseed oil was obtained from a supermarket in Guangzhou. $FeSO_4{\cdot}7H_2O$, Fe_2 $(SO4)_3{\cdot}xH_2O$ and Methanol (≥98%) was purchased from Tianjin Fuyu Fine Co. Free lipase (CALB) from the genetically modified *Candida antarctic* was donated by Novozymes-Denmark. All other chemicals and reagents used were of analytical grade and used as such without further purification and obtained from local market. Aqueous solutions were prepared in deionized water.

2.2. Preparation of Fe_3O_4 magnetic nanoparticles

Fe_3O_4 magnetic nanoparticles were prepared by co-precipitation method. Briefly, $FeSO_4{\cdot}7H_2O$ and Fe_2 $(SO_4)_3{\cdot}xH_2O$ were dissolved in [BMIN]BF_4 (0.25 mol/L) deionized water with 1:1 ratio to make Fe^{2+} (0.5 mol/L) and Fe^{3+} (1.0 mol/L). Then poured 20% strong ammonia water with stirred heavily under nitrogen at 70 °C till the pH reached 12.0. After 10 min of reaction, black Fe_3O_4 sediment was formed. The compound was collected by external magnetic field and washed with deionized water and absolute ethyl alcohol for several times. The product thus obtained was dried in a vacuum oven at 60 °C and stored for future use.

2.3. Functionalization of Fe_3O_4 magnetic nanoparticles with APTES

Fe_3O_4 magnetic nanoparticles (2 g) were dispersed in 25 mL of ethanol through ultrasonication for 30 min. APTES (0.5 g) solutions were added into the dispersed particles. The mixture was dispersed by ultrasonication sufficiently and then replaced in the shaker for shaking overnight at 30 °C. The obtained functionalized magnetic nanoparticles (APTES-Fe_3O_4) was collected by external magnetic field and rinsed with deionized water and ethanol to remove the excess organosilane reagents.

2.4. Lipase immobilization

For preparation of lipase-bound magnetic nanoparticles, 200 mg of glutaraldehyde activated functionalized Fe_3O_4 magnetic nanoparticles (2 g) was first dispersed in 25 mL of phosphate buffer (0.1 M phosphate buffer, pH 7.0), Then, the mixture was shaking at 30 °C for 2 h. Finally, the synthesized magnetic nanoparticles were washed well with deionized water to remove the unattached glutaraldehyde solution. The particles obtained from above mentioned process were applied for lipase immobilization. 25 mL of buffer solution (0.1 M phosphate buffer, pH 7.0) containing 800 mg of lipase were mixed with the above activated nanoparticles (2 g). The mixture was then shaken at room temperature for 2 h. The unbounded lipase was removed under a magnetic field, and the precipitate was recovered and washed carefully with phosphate buffer (0.1 M phosphate buffer, pH 7.0) for several times and then directly used for the lipase activity measurements. The pathways route of lipase immobilization on the surface of Fe_3O_4 magnetic nanoparticles is shown in Fig. 1.

2.5. Lipase assay

The enzymatic activities of immobilized and free lipase were tested with rapeseed oil emulsion containing 3% (w/v) PVA. A certain amount of the free or immobilized lipase was added to 4 mL of the emulsion and 5 mL of the phosphate buffer (0.025 M, pH 7.0). The hydrolysis reaction was carried out at 45 °C for 15 min. The quantity of fatty acid liberated was measured by titration with 0.1 M KOH solution. One unit (1 *U*) of activity was defined as the amount of lipase that liberates 1 μmol of oleic acid per minute under the assay conditions. As a reference test, titration with 0.1 M KOH solution was also performed for the as prepared nanoparticles after the washing step [34].

2.6. Characterization of magnetic nanoparticles

The crystal structure of the magnetic nanoparticles was identified by powder X-ray diffraction (XRD) using a PAnalytical X'Pert PROMPD diffractometer. The XRD atlas was matched with X' pert high score software. The scanned area was 2θ = 15°–90°, operated at 40 kV and 40 mA using Cu-Kα radiation. Fourier-transform infrared (FT-IR) spectra were collected on a Thermo Finnegan Nicolet 6700 FT-IR infrared spectrometer from 4000 to 400 cm^{-1} using the KBr pellet technique. Transmission electron microscopy (TEM) was performed using Philips-FEI Tecnai G2 F30 S-Twin microscope at 200 kV, equipped with an energy dispersive spectrometer (EDS) was conducted to further verify the morphology, size and the composition of the magnetic nanoparticles. The magnetic properties were measured using a SQUID magnetometer (QuantumDesign MPMS-XL) at room temperature.

2.7. Immobilized lipase mediated transesterification reaction

Transesterification reactions were carried out at 45 °C in a 50 mL capped flask on a shaking incubator with stirring rate of 250 rpm. A typical reaction mixture consisted of 10 g rapeseed oil, a weighed amount of the immobilized lipase, and a three-step addition of some amount of methanol in each step. Once the transesterification reaction had completed, the immobilized lipase was separated using a magnetic field from the reaction mixture, glycerol was separated by centrifugation, and the top layer was distilled under vacuum to eliminate excess methanol.

The Biodiesel (fatty acid methyl esters) was analyzed using a Shimadzu Gas Chromatograph (GC-2010) equipped with the AOC-20i automatic injection port and a flame ionization detector (FID). The capillary column was a DB-WAX (30 m × 0.25 mm × 0.25 μm); Methyl heptadecanoate, n-hexane, and argon were used as the internal standard, solvent, and carrier gas, respectively. Test conditions: He carrier gas, flow rate 1.0 mL/min, H_2 flow rate 40 mL/min; Air velocity 30 mL/min; temperature at sample injection port 280 °C; detector temperature 300 °C; programmed temperature, with an initial temperature of 80 °C, rising to 250 °C at a rate of 12 °C/min, kept for two minutes; split sampling, with split ratio of 1:20, sample size was 1μL.

3. Results and discussion

3.1. TEM-EDS analysis

In order to assess the magnetic nanoparticles morphology, size and the composition, TEM-EDS images were collected. The magnification TEM image of the Fe_3O_4 magnetic nanoparticles was shown in Fig. 2a, b. TEM images revealed that Fe_3O_4 magnetic nanoparticles were almost spherical or ellipsoidal with a mean particle size of 195 nm, and the particle size varied from 150 nm to 220 nm, some have some overlap, but they are mostly single-layer, the particle surface was comparatively smooth.

TEM micrographs of amino-silane modified Fe_3O_4 nanoparticles (APTES-Fe_3O_4) were presented in Fig. 2c, d. TEM images were clearly