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Fabrication of Magnetic Nano Enzyme Fe_3O_4-CS-Lipase and Testing its Catalytic Activity for Triglyceride Tranesterification Reaction with Methanol in Biodiesel Production

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Abstract: ***This work demonstrates herein a method to fabricate Fe_3O_4-CS-lipase NPs for triglyceride transesterification reaction and test its catalytic activity via reaction of soybean oil with methanol. The magnetic portion Fe_3O_4NP was synthesized from the mixture of Fe^{2+} and Fe^{3+} in alkaline medium using co-precipitation method, which was later coated with chitosan and then functionalized with glutaraldehyde as a cross-linking agent for lipase immobilization. Physical characterization by Fourier transform infrared spectroscopy (FT-IR), magnetic saturation, X-ray powder diffraction (XRD) showed that lipase was successfully immobilized on Fe_3O_4-CS NPs and the immobilized enzyme Fe_3O_4-CS-lipase NPs still showed good magnetic performance with a magnetic saturation value of 34.2 emu.g^{-1}. Transmission electron microscopy (TEM) image a revealed spherical shape of Fe_3O_4-CS-lipase NPs with size in the range of 17 ÷ 28 nm. Using the immobilized lipase based catalyst, the conversion of soybean oil to fatty acid methyl esters was successfully performed under the reaction temperature of 45^{o}C, reaction duration of 36 hours, molar ratio of methanol: oil of 3:1, and n-hexane as a co-solvent.***

Keywords: Magnetic nanoparticles, immobilized lipase, enzymatic transesterification, biodiesel, fatty acid methyl esters

1. Introduction

The search for alternative fuels has attracted attention during the past decade because of the inevitable depletion of fossil fuels. Biodiesel, a mixture of mono-alkyl esters derived from renewable feedstock, such as soybean oil, jatropha oil, rapeseed oil, palm oil, sunflower oil, corn oil, peanut oil, canola oil, and cotton-seed oil, is one of the most promising alternative fuels [1]. Considering the biodegradable, non-toxic, and almost sulfur-less characteristics, biodiesel is one of the most promising alternative fuels which can be used alone or be blended with conventional petro-diesel in unmodified diesel engines [1]. Most common biodiesel blends are B2 (2 % biodiesel, 98 % diesel), B5 (5 % biodiesel, 95% diesel), and B20 (20 % biodiesel, 80 % diesel) [2, 3].

Biodiesel is commercially produced through a chemical process called transesterification. In this process, oil and fat are transesterified after being mixed with short-chain alcohols, such as methanol or ethanol, in the presence of a catalyst. Therefore, the search for appropriate catalysts for transesterification has attracted attention of scientists. Until recent years, various kinds of catalysts have been utilized. The conventional catalysts are homogeneously strong bases or acids [4- 6]. These catalysts have the advantages of short reaction time, high yield, and low cost, but drawbacks also exist as the basic catalysts easily react with free fatty acids to form interfering soaps as hard-to-separate byproducts [7, 8]. Homogeneous acid catalysts operate at high temperatures, generally causing corrosion to equipment, and the reaction rate is observed to be low [9 -11].

Lipases (triacylglycerol hydrolase, E.C.3.1.1.3) are widely used in industry for catalyzing a variety of reactions, such as hydrolysis, alcoholysis, esterification, and transesterification [12] and as a result, biodiesel can be chemically synthesized using lipase catalyzed transesterification without encountering aforementioned drawbacks of the alkali/acid catalyzed processes. In addition, the enzymatic process can be performed with low quality oil feedstock containing high level of free fatty acids which can be directly converted to biodiesel; it also requires less energy consumption and the glycerol formed is easier to separate. However, free lipase-catalyzed reactions have various limitations, such as ease of lipase aggregation in the reaction medium and difficulty in two-phase separation and lipase recovery. In order to solve these problems, the enzyme is embedded on a suitable support through immobilization. This is a cost effective approach to keep the enzyme stable over time and to facilitate enzyme reuse.

Various techniques have been applied to lipases immobilization. Adsorption is a physical attachment of lipases onto the surface of support by weak forces, such as van der Walls, ionic and hydrophobic interactions, or dispersion forces [13]. Materials, such as phyllosilicates, accurel MP1004 (porous polypropylene), mesoporous silica, and silica zeolites have been reported [14- 17]. The physical adsorption technique is facile. However, desorption of enzyme is a common problem. Lipases can also be entrapped or encapsulated in a matrix of polymers like phyllosilicate sol-gel [18], cellulose acetate gel fiber [19], and silica gels [20- 23], chitosan [24- 26]. The immobilization procedures of entrapment and encapsulation are often simpler than those of covalent linkages, but the polymeric matrices often have

significant mass transfer resistance.

The production of biodiesel using immobilized lipase has been studied extensively. However, the processes of lipase immobilizations were done with meso porous bulk materials, resulting in a low reaction rate and low yield due to mass transfer resistance. In this work, lipase was covalently attached onto chitosan-coated Fe_3O_4 nanoparticles as a support for immobilization. With a high ratio of surface area to volume and valuable magnetic property, the synthesized Fe_3O_4-CS NPs allow high enzyme loading, low mass transfer resistance, and selective separation from the reaction mixture under an external magnetic field. These properties would help minimize the operation cost and enhance the product's purity. The catalytic activity of the synthesized Fe_3O_4-CS-lipase NPs was also tested through transesterification of soybean oil with methanol for biodiesel production.

2. Experimental

2.1 Materials

Ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), chitosan from shrimp shells (≥75% deacetylated), glutaraldehyde solution 25% were obtained from Sigma-Aldrich; methanol was purchased from Merck. Lipase from porcine pancreas (≥20,000 units/mg protein) was also supplied by Sigma-Aldrich. All other chemicals were of analytical grade and used without further purification. Commercial soybean cooking oil used in the transesterification was supplied by Tuong An cooking oil company, Vietnam. All solutions used in the experiments were prepared with distilled water.

2.2 Methods

Synthesis of Fe_3O_4NPs using hydrothermal method

Fe_3O_4NPs were prepared by co-precipitating a solution containing Fe^{2+} and Fe^{3+} ions by ammonia solution under hydrothermal conditions. Ferrous and ferric chlorides (molar ratio 1:2) were dissolved in water at a concentration of 0.1 M iron ions. Chemical precipitation was accomplished at 25°C under vigorous stirring by adding dropwise NH_4OH solution (25%) under protection of argon. During the reaction process, the pH was maintained at approximately 10. After mixing the solutions, the color of the solution changed from light brown to black, indicating the formation of Fe_3O_4NPs, which was allowed to crystallize completely for another hour under rapid stirring at 80°C. The products (Fe_3O_4NPs) were also washed by repeated cycles of centrifugation and redispersion in distilled water. Then, at room temperature, the final products were dried in a vacuum oven for 24 hours and kept for use.

Preparation of chitosan coated Fe_3O_4NP

Fe_3O_4NPs (1 g) were activated with pure ethanol twice, and then dispersed into 30 ml solution of chitosan in 1% acetic acid. The suspension was mixed by ultrasonic irradiation for 1 hour, and transferred into a beaker under vigorous stirring at room temperature after adding 3 ml 25% glutaraldehyde solution. After 3 hours, the chitosan-coated Fe_3O_4 nanoparticles (Fe_3O_4-CS NPs) were collected from the reaction mixture by external magnet and washed three times with water to remove unbound chitosan. The product Fe_3O_4-CS NPs was finally dried at 50°C in a vacuum oven.

Immobilization of lipase onto Fe_3O_4-CS NPs

Crosslinking with glutaraldehyde was utilized for lipase immobilization. First, 20 mL of glutaraldehyde (10%) in phosphate buffer saline (pH = 7,4) was added to 2 g of Fe_3O_4-CS NPs, stirred for 2 hours and incubated overnight at room temperature. The precipitate was then washed three times with distilled water and dried. For lipase immobilization step, 1000 mg of lipase was first added to 100 mL of phosphate buffer solution, pH = 7.4 under gentle stirring until all the lipase dissolved. Later, 2 g of Fe_3O_4-CS NPs activated with glutaraldehyde was added to the lipase solution and stirred for 24 hours at room temperature. The lipase immobilized was separated by external magnetic field and washed twice with phosphate buffer pH = 7.4 to remove unbound lipase. Finally, the immobilized lipase on Fe_3O_4-CS NPs (Fe_3O_4-CS-lipase NPs) was dried at room temperature in vacuum and stored at 4°C for later use.

Immobilization of lipase onto Fe_3O_4-CS NPs

Crosslinking with glutaraldehyde was utilized for lipase immobilization. First, 20 mL of glutaraldehyde (10%) in phosphate buffer saline (pH = 7,4) was added to 2 g of Fe_3O_4-CS NPs, stirred for 2 hours and incubated overnight at room temperature. The precipitate was then washed three times with distilled water and dried. For lipase immobilization step, 1000 mg of lipase was first added to 100 mL of phosphate buffer solution, pH = 7.4 under gentle stirring until all the lipase dissolved. Later, 2 g of Fe_3O_4-CS NPs, activated with glutaraldehyde, was added to the lipase solution and stirred for 24 hours at room temperature. The immobilized lipase was separated by external magnetic field and washed twice with phosphate buffer pH = 7.4 to remove unbound lipase. Finally, the immobilized lipase on Fe_3O_4-CS NPs (denoted as Fe_3O_4-CS-lipase NPs) was dried at room temperature in vacuum, stored at 4°C for later use.

Physical characterizations

The naked Fe_3O_4NPs and their composites were physically characterized by FT-IR analysis, X- ray diffraction analysis, TEM, and magnetic saturation measurements.

The IR spectra were recorded in the range 4000 to 400 cm^{-1} using KBr pellets on a Nicolet iS10 FT-IR spectrometer. The powder X-ray diffraction scans of samples were performed on a Bruker D8 Advance Diffractometer using Cu-Kα radiation (40 kV, 40 mA) with continuous scanning mode (2θ) from 5° to 80° at a rate of 0.02° min^{-1}. The samples for TEM images were suspended in ethanol. A drop of the sample was deposited on a carbon copper grid as a TEM support, and then dried under reduced pressure for 2 hours at room temperature. TEM characterization was carried out on a JEOL JEM-1200EX with accelerating voltage of 200 kV. A vibrating sample magnetometer (JDM-13D magnetometer) was used to characterize the magnetic properties (hysteresis of the magnetization) of Fe_3O_4NPs and their composite using a MPMS-XL 7 magnetometer at 300 K.

Transesterification procedure

The transesterification reaction was carried out in a 250 mL three-neck spherical glass reactor with condenser. A mixture of 10 gram soybean oil and 2.5 gram n-hexane was added into the reactor and mixed well. As the reactor reached the established temperature at 45°C, 1 gram of immobilized enzyme was added. An amount of 1.46 gram methanol was added into the mixture under stirring in three separate additions at 0, 12, 24 hours. The reaction mixture was stirred and refluxed for 36 hours. Fe_3O_4-CS-lipase NPs was separated from the reaction mixture by applying a strong external magnetic field. The reaction product was allowed to stand in a separating funnel for glycerol removal, followed by washing the collected crude methyl esters several times with hot distilled water (50°C) and eliminating water in the product by heating at 110°C.

Analytical procedure

The methyl ester contents were quantified using a gas chromatograph Agilent 6890N connected to a forte BP-20 capillary column (0.25 mm × 30 m) from SGE. The temperature program was as follows: 155 ºC for 1 minute and programmed from 155 to 180 ºC at a rate of 2 ºC/min, kept for 2 minutes, and finally raised to 220 ºC at 4 ºC/min and maintained for 6 minutes. The injector was set up for 250 ºC and the flame-ionization detector (FID) at 280 ºC. Nitrogen was used as carrier gas at constant flow of 1.6 mL/min. Methyl heptadecanoate in hexane was used as an internal standard.

3. Results and discussion

Fabrication of Fe_3O_4-CS-lipase NPs

The morphology and size of the synthesized Fe_3O_4NPs, Fe_3O_4-CS NPs was observed from TEM micrographs as shown in Figure 1. It is clear that the naked Fe_3O_4NPs were almost mono-dispersed with diameter in the range of 12 ÷ 17 nm (figure 1a), meanwhile their Fe_3O_4-CS NPs have a larger size of approximately 20 nm in diameter (figure 1b). This reveals that the coating process did not significantly result in the agglomeration of Fe_3O_4 NPs, which may cause considerably change to the larger size. There is a little aggregative phenomenon observed in the Fe_3O_4NP-CS sample. Thus, the chitosan coating occurred only on the Fe_3O_4 NP surface and our attempt to fabricate mono-dispersed chitosan-coated Fe_3O_4NPs for lipase immobilization in this work has been achieved.

XRD patterns for the three samples, including Fe_3O_4 NPs, Fe_3O_4-CS NPs and Fe_3O_4-CS-lipase NPs, were shown in the Figure 2 (a, b, c), respectively. Six characteristic peaks at 2θ values of 30.1°, 35.5°, 43.1°, 53.4°, 57.0°, and 62.6° were assigned to (220), (311), (400), (422), (511), and (440) reflections were observed for all analyzed samples. These peaks were consistent with the standard Fe_3O_4 (cubic phase) XRD spectrum. The outcome also illustrates that the resultant nanoparticles were pure Fe_3O_4 in a spinel structure, and the crystalline structure of the Fe_3O_4 NPs did not change during modification processes.

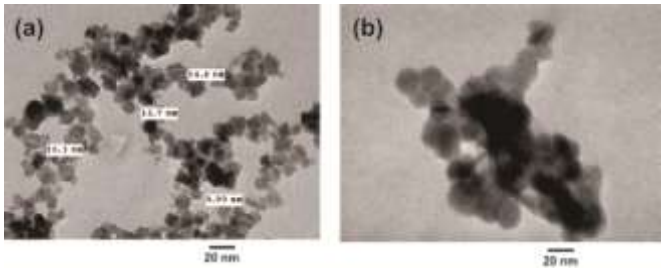

Figure 1: The TEM micrographs for the naked Fe_3O_4NPs (a) and Fe_3O_4-CS NPs (b).

To elucidate the mechanism and binding efficiency of chitosan onto Fe_3O_4 NPs, as well as chemical immobilization of enzyme lipase onto Fe_3O_4-CS NPs, FT-IR spectra of the Fe_3O_4-CS NPs (a) and Fe_3O_4-CS-lipase NPs (b) were examined as shown in Figure 3.

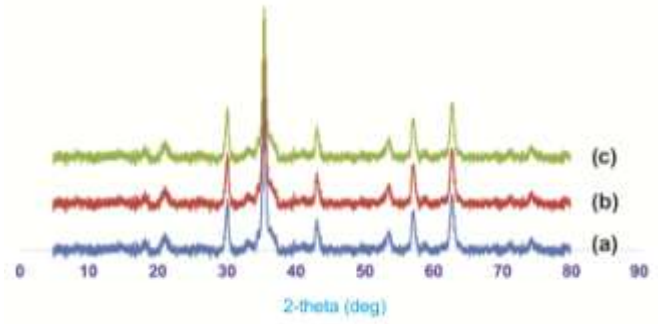

Figure 2: XRD patterns of Fe_3O_4 NPs (a), Fe_3O_4-CS NPs (b), and Fe_3O_4-CS-lipase NPs (c)

The peak around 3410 cm^{-1} observed in two spectra relates to the –OH group. For the IR spectrum of Fe_3O_4-CS NPs, the characteristic absorption bands appeared at 1641 cm^{-1} and 1399 cm^{-1} could be assigned to N–H bending vibration, and –C–O– in chitosan, respectively. The peak that appeared at 579 cm^{-1} relates to Fe–O vibrations of Fe_3O_4. The results indicated that chitosan was successfully coated onto Fe_3O_4 NPs by electrostatic interaction between the negative charges of iron oxide and positive charges of protonated chitosan with the aid of glutaraldehyde as a cross-linking agent.

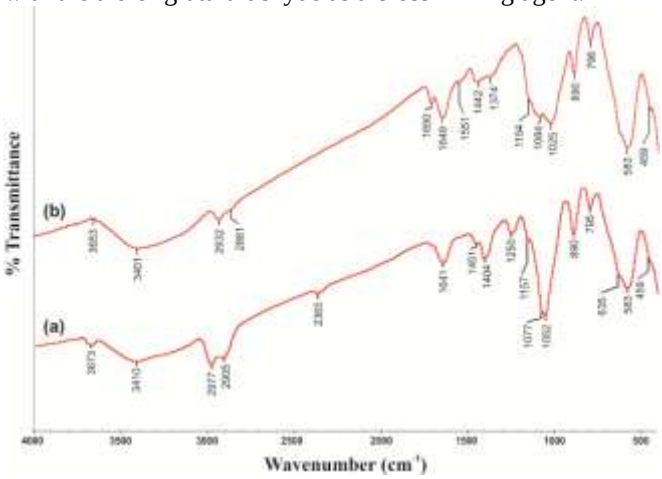

Figure 3: IR spectra of Fe_3O_4-CS NPs (a) and Fe_3O_4-CS-lipase NPs (b)

The IR spectrum of the Fe_3O_4-CS-lipase NPs, compared with that of the Fe_3O_4-CS NPs, appeared a new sharp absorption peak at 1690 cm^{-1} which can be assigned to C=N bond,

indicating reaction between $–NH_2$ group of the protein - lypase with carbonyl group of glutaraldehyde to form Schiff base [28].

Magnetic behavior of the synthesized materials plays an important role in separation and reuse of the Fe_3O_4NP based immobilized lipase. When superparamagnetic materials are high, they can be separated from the reaction mixtures more easily. The magnetic properties of the Fe_3O_4 NPs and their composite were measured at room temperature using a vibrating sample magnetometer (VSM). The hysteresis loops of the samples are shown in Figure 4. The saturation magnetization (σ_s) value of the bare Fe_3O_4NP was about 73.75 emu.g^{-1}, which represents a magnetic content of 88.64% compared to that of the pure magnetite nanoparticles of 83.2 emu.g^{-1}. After functioning and enzyme fixing, the saturation magnetizations for Fe_3O_4-CS NPs and Fe_3O_4-CS-lipase NPs were found to be 49.03 emu.g^{-1} and 38.29 emu.g^{-1}, respectively. As known, any crystalline disorder within the surface layer may lead to a significant decrease in the saturation magnetization of the magnetic NPs, thus, the decrease in saturation magnetization observed for Fe_3O_4-CS NPs and Fe_3O_4-CS-lipase NPs demonstrates the success of chitosan coating and lipase immobilization on the Fe_3O_4 NPs carrier.

To further investigate the performance of Fe_3O_4-CS-lipase NPs under external magnetic field, an amount of 0.5 g of Fe_3O_4-CS-lipase NPs was dispersed in 10 ml water in a glass bottle, resulting in a dark brown dispersion. Next, a permanent magnet was placed on the bottle wall to test for Fe_3O_4-CS-lipase NPs separation. It was found that the Fe_3O_4-CS-lipase NPs were completely aggregated to the magnetic applied area and the dispersion became clear and transparent in approximately two minutes as shown in Figure 5. This magnetic property helps to separate the magnetically immobilized lipases away from the product for multiple uses.

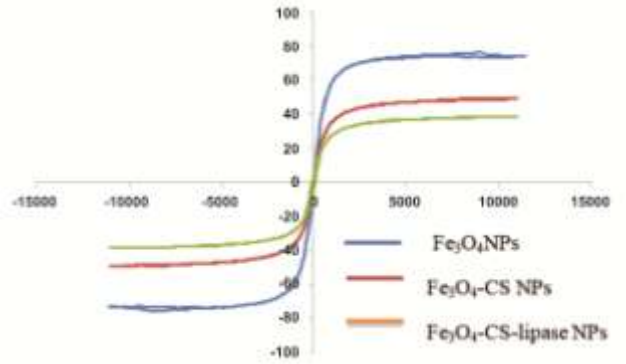

Figure 4: Hysteresis loop of magnetic Fe_3O_4NP (a), Fe_3O_4-CS NP (b), and Fe_3O_4-CS-lipase NP (c).

Figure 5: Images of Fe_3O_4-CS-lipase NPs before (a), and after being applied external magnetic field (b)

Transesterification reaction using Fe_3O_4-CS-lipase NPs as a catalyst

For enzymatic triglyceride transesterification reaction, main factors which are found to influence the conversion efficiency include reaction temperature, solvent for dispersion and molar ratio of triglyceride to methanol.

The higher yield will be possible if methanol is excessively compared to the molar stoichiometric ratio of 1:3 of lipid: alcohol, since the transesterification reaction is reversible. However, for enzyme catalyzed transesterification, insoluble leftover methanol which exists as fine droplets demonstrates negative effects on lipase activity and also decrease the production yield [29]. In this work, two useful solutions to prevent degradation of lipase catalytic activity were utilizing n-hexane as a co-solvent in order to increase the solubility of alcohol, and adding alcohol into the reaction mixture in three separate portions – one third of the total amount each time. Apart from that, the reaction temperature was controlled at 45^{o}C, creating catalytic activity of the immobilized enzyme and mass transfer efficiency to improve.

The oil phase of the reaction product was analyzed by GC-MS method as described earlier. The GC-MS chromatogram achieved, as shown in Figure 6, reveals six main types of methyl esters that were found as the final product. Comparing the diesel from crude oil, the average carbon chain is shorter, indicating the biodiesel production could have a higher combustion feature than petrol-diesel. The final methyl ester products can be blended with conventional petro-diesel as fuel in unmodified diesel engines.

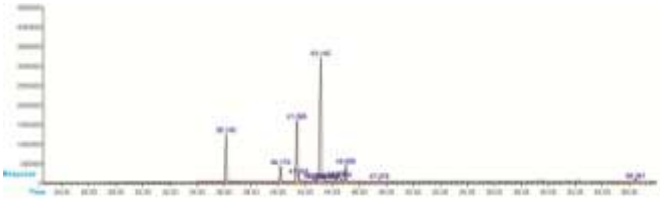

Figure 6: GC-MS chromatogram of the transesterification products of soybean oil with methanol catalyzed by Fe_3O_4-CS-lipase NPs.

Table 1: The composition of final products of transesterification of soybean oil with methanol at different retention times

No	*RT*	*Name of methyl esters*	*Percentage*
01	36.145	Palmitic acid methyl ester	13.841
02	40.175	Stearic acid methyl ester	5.195
03	41.385	Oleic acid methyl ester	20.987
04	41.524	-	1.542
05	42.642	-	0.815
06	42.818	-	0.652
07	43.142	Linoleic acid methyl ester	46.731
08	43.466	-	0.535
09	43.848	-	0.436
10	44.308	-	1.154
11	44.704	-	1.039
12	45.005	Linolenic acid methyl ester	4.976
13	47.476	-	0.484
14	66.461	-	1.614
In total		**Methyl esters**	91,730

* - : undetermined

4. Conclusion

In this work, we showed a useful method to fabricate the magnetic nano enzyme, Fe_3O_4-CS-lipase NPs, as a bio-catalyst for biodiesel production through transesterification of soybean oil with methanol. Considering the nano size of Fe_3O_4-CS-lipase NPs, the fabricated magnetic enzyme can speed up the reaction rate as well as the homogeneous free lypase. The immobilized lipase exhibited a good catalytic activity at 45°C under three separate additions of methanol at 0, 12, and 24 hours and use of n-hexane as a co-solvent to minimize the lipase catalytic activity lost. With high saturation magnetization value of 38.29 emu.g^{-1}, the catalyst can be easily recovered for multiple uses by an applied external magnetic field.

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