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Modification of magnetic nanoparticle lipase designs for biodiesel production from palm oil

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ABSTRACT

Biocatalytic conversion of vegetable oils by immobilized lipase to fatty acid methyl ester (FAME) is an efficient eco-friendly alternative to the conventional alkaline-catalyzed biodiesel production process. In this work, immobilization of *Thermomyces lanuginosus* lipase on Fe_3O_4 was studied using different covalent linkage designs. Immobilization of lipase on magnetic supports was shown by Fourier-Transformed infrared microscopy and scanning electron microscopy. Immobilized lipase prepared on Fe_3O_4 carrier modified by 3-aminopropyl triethyoxysilane and covalently linked by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide (Fe_3O_4-AP-EN-LIP) showed the highest catalytic activity on hydrolysis of *p*-nitrophenyl palmitate and transesterification of refined palm oil. Reaction variables were optimized by Central Composite Design, which identified 23.2% w/w enzyme loading and 4.7:1 methanol to FFAs molar ratio with 3.4% water content in the presence of 1:1 (v/v) *tert*-butanol to oil as optimal conditions, leading to 97.2% FAME yield after incubation at 50 °C for 24 h. The biocatalyst showed high operational stability and could be simply separated by magnetization and recycled for at least 5 consecutive batches with >80% activity remaining, suggesting its potential for application in biocatalytic biodiesel synthesis.

1. Introduction

Biodiesel is an alternative renewable fuel to petroleum diesel. It is produced as fatty acid alkyl esters, which are mostly obtained by transesterification or esterification of glycerides and fatty acid in vegetable oils with short chain alcohols. At present, biodiesel accounts for around 10% of the world's diesel usage, and its share is expected to increase owning to fluctuation in petroleum price and environmental concerns. It possesses several technical and environmental advantages over conventional diesel such as higher cetane rating, less sulfur and carbon monoxide emission, lower unburned hydrocarbons and particulate matter, and can be blended with conventional petroleum diesel up to 20% with no engine modification [1].

Several chemocatalytic, thermocatalytic, and biocatalytic approaches have been explored for increasing the efficiency of biodiesel production, and for overcoming drawbacks of the currently used alkaline-catalyzed process, which is sensitive to free fatty acid in crude feedstock and requires high chemical usage with costly downstream processing [2,3]. The lipase-catalyzed enzymatic process has many advantages for the synthesis of alkyl esters over the alkaline-catalyzed reaction, including ability to convert both glycerides and free fatty acids to esters under mild operating conditions, simple downstream processing with easy recovery of glycerol, and no requirement for subsequent wastewater treatment. Immobilization offers a promising approach for improving enzyme operational stability in non-aqueous systems and allows reusability of the biocatalyst. Various techniques have been used for lipase immobilization e.g. adsorption [4], covalent bonding [5], entrapment [6,7], enzyme crystal [8], and microcrystalline enzymes e.g. protein-coated microcrystals (PCMCs) [9] and cross-linked PCMCs (CL-PCMCs) [10,11].

Immobilization of enzymes requires solid carriers, which influence the physico-chemical characteristics and biological activity of immobilized enzymes. Recently, several types of micro-/nano-structure magnetic supports have been extensively studied as solid matrices for enzyme immobilization, which allow fast and facile separation of products from enzyme under a magnetic field [12]. Magnetic nanoparticle immobilization of various enzymes e.g. laccase, α-amylase, β-galactosidase, and lipase also provides a large reactive surface area [13–15], and can improve enzyme stability [16]. Several forms of magnetic materials and covalent attachment methods have been tested for preparation of immobilized lipases using different designs, which provide immobilized enzymes that vary in their catalytic performance in aqueous and non-aqueous reactions. Immobilization of

Abbreviations: FAME, fatty acid methyl ester; MeOH, methanol; RPO, refined palm oil; FFAs, free fatty acids; *t*-BuOH, *tert*-butanol

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lipase on Fe_3O_4 nanoparticle treated with (3-aminopropyl) triethoxysilane (APTES) using glutaraldehyde as a coupling reagent was first reported for transesterification of soybean oil [17]. Composite inorganic or biological polymers can improve magnetic nanoparticle-immobilized lipase stability by strengthening the core magnetic structures or modifying physical properties of the magnetic carriers e.g. chitosan-coated Fe_3O_4 nanoparticle [18], magnetic composite poly(styrene-methacrylic acid) microsphere [19], alkyl-functionalized Fe_3O_4-SiO_2 [15], and Fe_3O_4/SBA-15 composite [20]. These magnetic immobilized lipases were applied for catalyzing various reactions, for examples, biodiesel production by transesterification and esterification [17–19] and interesterification of oils in food industry [20].

Although several designs for immobilization of lipases using "bare" and "composite" magnetic nanoparticles have been reported, they are still not economically attractive owing to inadequate catalytic performance of the immobilized enzyme, which is reflected in the high enzyme dosage (>40% w/w) needed to achieve a feasible FAME yield and low operational stability [17,19,21] In this study, various designs of magnetic nanoparticle lipase for biodiesel synthesis were explored using different covalent modification methods for attaching the enzyme to a core-magnetic matrix in order to improve the enzyme's catalytic performance and stability. The physicochemical characteristics of enzyme immobilized to different matrices were analyzed and the catalytic performance on transesterification of refined palm oil (RPO) was evaluated. Effects of key reaction factors were optimized for the best nanoparticle-lipase using the central composite design for maximizing product yield. The optimized process is described for preparation of highly active magnetic nanoparticle lipase with catalytic performance superior to previous designs, which has greater potential for eco-biodiesel industry.

2. Materials and methods

2.1. Materials

Refined edible grade palm oil (palm olein; RPO containing > 99% TAG) was obtained from a local market. Fatty acid ester standards were obtained from Sigma-Aldrich. Liquid *Thermomyces (Humicola) lanuginosus* lipase (Lipolase 100 T) was purchased from Novozymes (Bagsvaerd, Denmark). Chemicals and reagents for immobilized lipase preparation (3-aminopropyl triethoxysilane (APTES), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), and glutaraldehyde (GA) were purchased from Sigma-Aldrich. Chemicals were of analytical grade and obtained from major chemical suppliers (Sigma-Aldrich, Merck, and Fluka). All reagents on transesterification were dehydrated with 3 Å molecular sieves (Fluka, Buchs, Switzerland) before use.

2.2. Preparation of magnetic nanoparticle support

Magnetic nanoparticle support (Fe_3O_4 nanoparticle) was prepared using the method modified from Xie and Ma in 2010 [22]. $Fe_2SO_4 \cdot 7H_2O$ (2.78 g) and $FeCl_3 \cdot 6H_2O$ (5.4 g) equivalent to a molar ratio of 1:2 were dissolved in 100 mL distilled water at a final concentration of 0.3 mol/L Fe^{2+}/Fe^{3+} combined ions. Iron nanoparticle was co-precipitated by drop-wise addition of 75 mL of 25 % ammonia hydroxide solution at 25 °C in a stirring vial. The suspension was incubated at 80 °C for 30 min and then cooled down to room temperature. The precipitate was separated by centrifugation at 5000 ×*g* and washed thrice with distilled water. The Fe_3O_4 nanoparticle was then dried in a dry oven at 60 °C.

2.3. Immobilization of lipase to magnetic nanoparticle

2.3.1. Type I: Fe_3O_4 nanoparticle lipase via EDC (Fe_3O_4-E lipase)

The Fe_3O_4 nanoparticle (0.2 g) was dispersed in 2 ml of 3 mM potassium phosphate buffer, pH 6 containing 100 mM NaCl. The suspension was added to 0.5 ml of 2.5 mg/mL solution of EDC and then sonicated at a frequency of 35 kHz for 15 min. The lipase solution (2.5 mL) was added to the suspension and sonicated at 30 kHz for 30 min. The biocatalyst was separated by magnetic decantation and then washed with the buffer for several times until no free lipase was detected in the supernatant as shown by Bio-Rad Bradford's assay reagent using bovine serum albumin (BSA) as a standard. The biocatalyst (Fe_3O_4-E lipase) was air dried and stored at 4 °C for subsequent use.

2.3.2. Type II: Fe_3O_4 nanoparticle lipase via EDC and cross-linked by GA (Fe_3O_4-E/G lipase)

The Fe_3O_4-E lipase (0.2 g) was treated with 0.2 mL of 0.5% v/v GA and 0.5 mL of 25 mM potassium phosphate buffer, pH 7 in the total reaction volume of 10 mL. The suspension was incubated at room temperature with shaking at 100 rpm for 2 h. The resulting nanoparticle lipase was separated by magnetization and washed thrice with distilled water to remove excessive GA. The biocatalyst was air-dried and stored at 4 °C for subsequent study.

2.3.3. Type III: Fe_3O_4-APTES nanoparticle lipase via EDC and NHS (Fe_3O_4-AP-EN lipase)

Fe_3O_4-modified by APTES was prepared using the method modified from Xie and Ma (2009) [17]. The Fe_3O_4 nanoparticle (0.25 g) was mixed with 0.15 mL APTES in 4.85 mL ethanol. The mixture was sonicated at a frequency of 35 kHz with shaking overnight at room temperature. The product was separated by magnetic separation, washed thrice with 5% ethanol and then dried in a dry oven at 50 °C to obtain Fe_3O_4-APTES. The lipase solution (2.5 mL) was mixed with 0.5 mL of 2.5 mg/mL EDC solution and incubated at room temperature for 2 h with shaking at 200 rpm. The mixture was added to 3 mg of NHS and further incubated under the same condition for 2 h. The solution was then added to 0.25 g of Fe_3O_4-APTES nanoparticle and further incubated for 2 h. The nanoparticle lipase was separated by magnetization and then washed with distilled water for several times until no free lipase was detected in the supernatant. The biocatalyst was air-dried and stored at 4 °C for subsequent study.

2.3.4. Type IV: Fe_3O_4-APTES nanoparticle lipase via EDC and NHS and cross-linked by GA (Fe_3O_4-AP-EN/G lipase)

The Fe_3O_4-AP-EN lipase (0.25 g) was crosslinked with 0.2 mL of 0.5% v/v GA and 0.5 mL of 25 mM potassium phosphate, pH 7 in a total reaction volume of 10 mL. The suspension was incubated at room temperature for 2 h with shaking at 100 rpm. The nanoparticle lipase was removed by magnetic separation and washed thrice with distilled water for removing excess GA. The biocatalyst was air-dried and stored at 4 °C for subsequent study.

The immobilization efficiency of lipase was determined according to Eq. (1).

$$\text{Immobilization efficiency}(\%) = \frac{(C_i - C_f) \times V_1}{C_i \times V_2} \times 100 \quad (1)$$

C_i and C_f represent the initial concentration of protein in the lipase solution before immobilization and the final concentration of protein in the supernatant after immobilization, respectively (mg/mL). V_1 and V_2 are the volume of the starting lipase solution and the final supernatant, respectively (mL).

2.4. Lipase activity assay

Lipase hydrolysis activity was assayed based on hydrolysis of *p*-nitrophenyl palmitate [23]. The standard reaction (200 μL) contained 20 mM sodium phosphate buffer, pH 8, 1 mM of *p*-nitrophenyl palmitate and an appropriate dilution of the enzyme or immobilized enzyme. The reaction was incubated at 45 °C for 30 min and then terminated by addition of 100 μL of 0.2 M Na_2CO_3. The formation of *p*-nitrophenolate

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