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Covalent immobilization of *Candida antarctica* lipase on core-shell magnetic nanoparticles for production of biodiesel from waste cooking oil

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ABSTRACT

In the present work, lipase from *Candida antarctica* (CALB) was covalently immobilized on functionalized magnetic nanoparticles (MNPs) to catalyze biodiesel synthesis. Core-shell nanoparticles were synthesized by coating Fe₃O₄ core with silica shell (Fe₃O₄@SiO₂). The nanoparticles functionalized with (3-glycidoxypropyl)trimethoxylsilane (GPTMS) were used as immobilization matrix. The protein binding efficiency on functionalized Fe₃O₄@SiO₂ was calculated as 84%, preserving 97% of specific activity of the free enzyme. Physical and chemical properties of the nanoparticles and the immobilized lipase were characterized by TGA, XRD, SEM, IR, TEM and DLS. Higher thermal stability and methanol tolerance for immobilized derivatives were obtained compared to the free enzyme. The immobilized lipase was then used to produce biodiesel by transesterification of waste cooking oil with methanol. In an optimization study, the effect of oil to methanol ratio, *tert*-butanol and molecular sieve as water adsorbent on the yield of biodiesel production were considered. Optimum oil to methanol ratio at 1:3 was observed for immobilized CALB in biodiesel production. Molecular sieve had a great effect on yield, with almost 100% conversion. The immobilized preparation of CALB also presented a good reusability, keeping 100% of its initial activity after 6 cycles of the reaction.

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1. Introduction

Exhaustion of fossil resources and their environmental effects are the main reasons for investigating on renewable fuels. Biodiesel is a biodegradable fuel that has received increasing attention in the recent years to use in compression—ignition engines [1]. It is defined as monoalkyl esters of long chain fatty acids including methyl and ethyl esters, derived from vegetable oils or animal fats as renewable feedstock [2]. Biodiesel has a similar engine performance and emits less harmful emissions in comparison with conventional fuel [3]. For example while biodiesel has almost zero sulphur content, the fossil fuels contain more sulphur and therefore damaging the atmosphere by releasing SO₂ [1]. Edible and non-

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edible oils have been recently reviewed as feedstock for biodiesel production [4]. The edible sources might face the fuel vs food dilemma as well as the deforestation issue. Therefore, the use of non-edible oils such as Jatropha curcas seed oil, yeast oil, castor oil, microalgae oil and waste oil for biodiesel synthesis is more promising than the use of edible oils [5–7]. Moreover it has been reported that the cost of raw materials is about 80% of the total biodiesel production cost which can be effectively reduced by the use of waste oils as a non-edible feedstock, in particular [8]. The raw material costs for waste cooking oils could be about one third of the price of virgin vegetable oils [9]. Moreover large amounts of waste cooking oils are produced annually throughout the world. Therefore its management is significant challenge because of environmental problems [1]. The amount of these low quality oils are over 15 million tons per year, which, if converted to biodiesel, satisfy the main part of the world demand [10]. However, the use of waste cooking oils as feedstock is rather challenging as it basically contains high content of free fatty acids (FFAs) and water





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concentrations which makes them unsuitable for the homogeneous alkaline-catalyzed trans-esterification as conventional biodiesel production process. Although conventional chemical technology using either acid or base chemical catalysts has been applied to biodiesel production, difficulties in glycerol recovery from biodiesel and removal of inorganic salts, high temperature and undesirable side reactions along with transesterification reaction are the certain limitations that restrict the use of chemical catalysts [11]. On the other hand utilization of lipases as a catalyst for biodiesel fuel production has great potential compared with chemical methods.

Lipases (E.C.3.1.1.3) are able to catalyze a variety of chemical reactions such as hydrolysis, esterification, transesterification, alcoholysis, and hence are widely used in industry [12]. There are also several reports on lipase-catalyzed production of biodiesel from oils [13–15]. Enzymatic production of biodiesel is normally affected by many factors such as lipase activity, stability and specificity, alcohol to oil ratio, temperature, and water content [1,11,16]. High efficiency, complete conversion of free fatty acids (FFA) to methyl/ethyl esters, less energy consumption, mild reaction conditions, high purity of the products and easy separation of the glycerol are the main advantages of using lipases in biodiesel production [17]. Furthermore, the enzymatic process is very compatible with low quality feedstocks with high levels of FFA. However, the commercialization of the enzymatic production of biodiesel remains problematic, because of high cost and low stability of lipases and also longer reaction times of these biocatalysts. To reduce the production cost of this process, strategies can be made specifically in up-stream processing in which the catalytic stability and recyclability of lipase can be improved by its immobilization on a solid support. Immobilization of enzymes has been investigated for many years, and different immobilization techniques such as crosslinking, adsorption, entrapment and encapsulation have been used [18–20]. Among all the strategies used for enzyme immobilization, covalent attachment meets the demand of industrial applications of biocatalysts [21]. This method has advantages of no leaching of enzyme from the support, wide choices of organic linkers and established methods of functionalization and modification of the supports [22].

In recent decades, magnetic nanoparticles have drawn great attention for practical application as support for enzyme immobilization due to their high surface area and proper physical properties [23]. The bare non-porous magnetic nanoparticles could be damaged due to erosion caused by unwanted interactions with reacting agents [24]. The grafting of silica layer on magnetic nanocores creates silica composite magnetic nanoparticles which can prevent the magnetic core from being eroded by environmental reaction as a protective shell [25]. Moreover the grafted nanoparticles can be easily functionalized with different functional groups to generate chemical bonds with enzymes during immobilization process [26]. There are several reports in literature reporting fabrication of superparamagnetic (Fe₃O₄) core and silica shell [27].

Here, we report the results of covalent immobilization of CALB on epoxy-functionalized silica-magnetic nanocomposite for the first time. Epoxy-functionalized supports are generally very stable at neutral pH values even in wet conditions and can be stored for a long time [28]. In fact because of these unique properties, these activated supports have been widely used for immobilization of different enzymes. Our previous experience also showed that the immobilized lipases on epoxy-functionalized silica were quite stable and reused for 16 cycles in biodiesel production without significant loss in activity [29]. Therefore the obtained nanocomposite grafted with epoxy groups was used as biocatalyst for the conversion of waste cooking oil with methanol to fatty acid methyl esters (FAMEs). Reusability of the immobilized lipase was also investigated by repeated-batch operations of the immobilized lipase-catalyzed transesterification of waste cooking oil with methanol. Furthermore several reaction parameters are studied here, aiming to obtain optimal conditions for the methanolysis of waste cooking oil. To the best of our knowledge, there is no previous report on immobilization of CALB on epoxy functionalized magnetic nanoparticles to use as biocatalyst for biodiesel production.

2. Experimental

2.1. Materials

Bovine serum albumin, triethylamine, para-nitrophenyl butyrate, (3-glycidyloxypropyl)trimethoxysilane (GPTMS), molecular sieve, tetraethyl orthosilicate (TEOS), iron(III) chloride hexahydrate, iron(II) chloride tetrahydrate, Candida antarctica lipase B and methyl ester standards were from Sigma. Waste cooking oil (with an initial saponification number of 196.2 mg KOH/g, acid value of 76 mg KOH/g corresponding to a free fatty acid (FFA) level of 38%) was obtained from a local restaurant with the following composition in fatty acids (w/v): 40.6% oleic acid, 17.6% linoleic acid, 32.2% palmitic acid, 5.2% stearic acid. The molecular weight of the oil which was calculated from its saponification value was determined to be 856.3 g/mol. Water content in the oil measured by Karl Fischer titration method was determined to be 0.01% (w/w). Dynamic laser scattering (DLS) was performed on Malvern ZEN 3600. Thermogravimetry (TGA) and differential thermal analysis (DTA) were carried out from 10 °C to 800 °C at a heating rate of 20 °C/min in air atmosphere using a STA 503M system from Bahr GmbH, Germany.

2.2. Synthesis of Fe_3O_4 nanoparticles

Chemical co-precipitation of Fe^{3+} and Fe^{2+} ions with a molar ratio of 2:1 was used for Fe_3O_4 nanoparticles synthesis [30]. Briefly, 3.40 g of $FeCl_3 \cdot 6H_2O$ and 1.25 g of $FeCl_2 \cdot 4H_2O$ were dissolved in 100 ml of deionized water under vigorous stirring. Then 6 mL of 25% NH₃·H₂O was added and vigorously stirred for 30–40 min at 60 °C. The solution instantly became black due to the formation of Fe₃O₄. The obtained nanoparticles were washed several times with deionized water and once with ethanol and then used directly for being coated with silica.

2.3. Preparation of silica-coated magnetic nanoparticles

For coating a silica layer on magnetic nanoparticles, hydrolysis of TEOS using the sol–gel process was performed in the presence of a constant flow of N_2 . For this, 0.145 g of Fe₃O₄ was added to 40 mL of ethanol and dispersed under ultrasonication for 10min. Then 6 mL of deionized water, 3 mL of ammonium hydroxide solution and 0.4 mL of TEOS were added to the suspension and stirred for 5 h at room temperature. Then the silica-coated nanoparticles were separated by a permanent magnet, followed by washing 3 times with ethanol and drying at 60 °C in vacuum for 24 h.

2.4. Functionalization of silica coated magnetic nanoparticles

To 50 mL of dry toluene 1 g of silica magnetic nanoparticles were added followed with adding 1.2 mL of GPTMS and 200 mL Et₃N. Then the mixture under nitrogen atmosphere and vigorous stirring refluxed for 4 h. The modified supports were washed with acetone for 3 times, collected by a permanent magnet and stored at 4 $^{\circ}$ C.

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