Energy Conversion and Management 159 (2018) 42–53

Contents lists available at ScienceDirect

Energy Conversion and Management

journal homepage: www.elsevier.com/locate/enconman

Immobilization of *Candida rugosa* lipase onto graphene oxide Fe_3O_4 nanocomposite: Characterization and application for biodiesel production

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ARTICLE INFO

Keywords:
Magnetic nanoparticle
Immobilized lipase
Transesterification
Graphene oxide
Vegetable oil
Biodiesel

ABSTRACT

The purpose of this work is to develop a magnetically recyclable immobilized lipase for biodiesel production to meet the need of green and clean production. To achieve this, magnetic Fe_3O_4 nanoparticles were encapsulated in graphene oxides (GO), and then employed as a magnetic carrier for the immobilization of lipase from *Candida rugosa* via the interfacial activation of enzyme on hydrophobic surfaces. By using this immobilization strategy, the activity recovery of 64.9% and enzyme immobilization efficiency of 85.5% could be achieved. The as-prepared graphene oxide Fe_3O_4 nanocomposite (MGO) and immobilized lipase microsphere were fully characterized by means of enzyme activity assays, transmission electron microscopy (TEM), X-ray powder diffraction (XRD), X-ray photoelectron spectroscopy (XPS), Fourier transform infrared (FT-IR) spectroscopy, vibrating-sample magnetometer (VSM) and nitrogen adsorption–desorption techniques. It was shown that the Fe_3O_4 nanoparticles were successfully encapsulated into the GO, and moreover the lipase was tethered on the magnetic support. The immobilized lipase could efficiently catalyze the transesterification of soybean oil with methanol for the production of biodiesel. With this magnetic biocatalyst, the biodiesel yield of 92.8% could be obtained at a temperature of 40 °C by three-step addition of methanol in a shaking water bath. The immobilized lipase could be easily recovered by using an external magnetic field, allowing for recycling of the biocatalyst five times without significant loss of its activity.

1. Introduction

Over the past decades, a great deal of effort has been devoted to the search for renewable energy alternatives to fossil fuels. Biodiesel, consisting of long-chain fatty acid methyl esters and as an alternative renewable energy, has fascinated a significant interest regarding the limited fossil fuel reserves and the intensified environment pollution [1,2]. Biodiesel production is generally carried out using catalytic transesterification of animal or vegetable oils with methanol. Homogeneous alkaline catalysts, such as NaOH or KOH are usually applied for biodiesel production due to their high activities under mild reaction conditions, but such liquid catalysts are hardly separated from the reaction mixture, and a large amount of waste water is generated during the costly and inefficient separation processes [3]. Besides, the alkali-catalyzed procedure commonly requires the use of raw oils with low contents of free fatty acid (FFA) and water, since the FFA can lead to the deactivation of base catalysts through the formation of soaps. More recently, a green approach for the transesterification processes has stimulated the development of recyclable solid catalysts as replacements for the liquid catalysts due to ever-growing environmental and economical concerns [4–6]. Among the heterogeneous catalysts, calcium oxide-based solid catalysts have attracted considerable interest because of their high basic properties, non-toxicity and low cost [7,8].

Lipases are the most widely used enzymes in biocatalysis, both at industrial and at academic level. In comparison with the chemical catalysts, lipase catalyst has several merits including milder reaction condition, little or no side reaction, environment friendly properties and lower susceptibility to the FFA and moisture present in the reactants. For example, *Candida rugosa* lipase is usually employed as a biocatalyst for the production of biodiesel, giving a high conversion to biodiesel [9]. However, the lipase-mediated transesterifications of vegetable oils have been hindered mainly due to the difficulties of enzyme recovery and the lack of long-term operational stabilities [10]. To solve these issues, great research interest has been devoted to the immobilization of lipase and the improvement of its application [11]. The immobilized lipase can greatly enhance the catalytic stability with ease of enzyme recovery after the reaction, thus contributing to the reduction in production cost. In this connection, several lipase immobilization strategies have been extensively investigated during the recent decades for the production of biodiesel, such as physical adsorption,

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https://doi.org/10.1016/j.enconman.2018.01.021
Received 30 September 2017; Received in revised form 6 January 2018; Accepted 9 January 2018

covalent binding, and hydrophobic ion pair affinity ligation [12,13]. Enzymes immobilized on non-porous nanomaterials are subject to minimum diffusion limitation though the enzyme loading per unit mass of support is usually low. In contrast, porous supports can afford high enzyme loading, but suffer a greater diffusion limitation of substrate [14]. To minimize the diffusion problem, a decrease in the particle size of the immobilized lipase is a good solution. Unfortunately, in this case the recovery of the biocatalysts is generally limited due to their small-size particles and less-sedimentation in the reaction mixture, and the inevitable loss in the separation process is still existed [15]. Therefore, there is urgent need of searching host matrix material for immobilized enzymes with improved catalytic performances [16].

Lipases exhibit a peculiar catalytic mechanism of action and have two conformations: a closed form where the active center is secluded from the medium by a polypeptide chain called lid or flat, and a open form where the lid is displaced and the active center of the lipase is exposed to the reaction media [17]. In the presence of hydrophobic surface, the open form of the lipase becomes adsorbed via the large hydrophobic pocket around the active center and the open form becomes stabilized, which is called the interfacial activation of lipases [18]. By using a proper immobilization method, some enzyme properties, such as stability, activity, selectivity and even purity, can be improved for the immobilized lipase [19]. Among the different supports for the immobilization of lipase, the utilization of hydrophobic carrier seems to be an efficient one, enabling the immobilization method to follow the interfacial activation mechanism and rendering the final biocatalysts to be stabilized in their open form [20,21].

Filtration and centrifugation are the commonly used method to separate the immobilized lipase from the liquid reaction mixture. Although the nano-sized immobilized lipases exhibited better catalytic efficiency thanks to their less mass diffusion limitations, their separation and recovery from the reaction mixture by filtration and centrifugation seem to become difficult [15,22]. A solution as applied to solve this issue is to develop an attractive alternative approach to conventional filtration or centrifugation for the nanocatalyst recovery. Very recently, magnetic nanoparticles, as a potent enzyme support, have received more attentions of researches, since the magnetite-loaded enzymes allow the easy recovery of the biocatalyst from the reaction mixture with minimal loss by using an external magnetic field [23,24].

However, the magnetic nanoparticles tend to aggregate into large clusters and lose the single-domain, thereof hindering the biocatalyst to be highly dispersed in the reaction mixture and reducing the catalytic efficiency [25,26]. It was reported that suitable passive materials such as silica, mesoporous MCM-41, carbon material (such as graphene oxide) or synthetic polymer can be encapsulated in magnetic nanoparticles in order to prevent their aggregations in the reaction mixture and improve their chemical stabilities [27–29]. Moreover, the thus-formed composites have their comprehensive benefits through the synergism of their individual components.

Graphene oxide (GO), as a graphene derivative acquired readily from natural graphite by strong oxidation and subsequent exfoliation, bears various oxygen-containing functional groups such as −COOH, −OH, C═O and epoxides on the surface [30,31]. These abundant surface functional groups on the GO can be the key chemical skeletons that are used as immobilizing sites for pure lipase, facilitating efficient lipase immobilization through chemical bonds or electrostatic interactions [32,33]. Besides, GO has demonstrated to have high chemical stability, good biocompatibility, great mechanical strength and large specific surface area. More importantly, lipase with primary amine groups can be readily immobilized on the surface of the GO with carboxyl groups by amidation reactions [32–34]. With this respect, grafting magnetic nanoparticles to GO surface not only can provide convenience for the solid-liquid separation, but also can afford an ideal magnetic carrier for the lipase immobilization [35].

In an attempt to develop an efficient and environmentally benign process for the production of biodiesel, in this investigation, the Fe_3O_4 nanoparticles were encapsulated in the GO, and the thus-formed magnetic graphene oxide (MGO) was then utilized as magnetic carriers for the immobilization of lipase through amide linkages by applying 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as an activating agent. The integration of magnetic nanoparticles into the GO can combine the high surface area of heterogeneous nature and the separation convenience of magnetic properties. The successful immobilization of lipase was confirmed by enzyme activity assays, X-ray photoelectron spectroscopy (XPS) and Fourier transform infrared (FT-IR) spectroscopy. The morphology and magnetic behaviors of the MGO before and after lipase binding were characterized by using transmission electron microscopy (TEM), vibrating-sample magnetometer (VSM), X-ray powder diffraction (XRD), magnetic measurements, and nitrogen adsorption–desorption techniques. By using this magnetic biocatalyst, the transesterification of soybean oil was carried out in a heterogeneous manner in a shaking water bath for the production of biodiesel. The operational stability and reusability of the immobilized lipase was also evaluated in the current research.

2. Materials and methods

2.1. Materials

Commercial liquid *Candida rugosa* lipase, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysulfosuccinimide (NHS) were purchased from Sigma-Aldrich. Commercial soybean oil, with an average molecular weight of the triglycerols of 874 g/mol, was obtained from a local grocery store (Zhengzhou, China), having the following fatty acid composition: 12.3% palmitic acid, 5.8% stearic acid, 26.5% oleic acid, 49.4% linoleic acid, and 5.9% linolenic acid. Graphite powder was provided by Tianjin Kaitong Chemical Factory (Tianjin China). Other materials used were analytical grade and obtained from a local chemical supply companies (Zhengzhou, China).

2.2. Preparation of magnetic graphene oxide composites

Graphene oxide (GO) was synthesized by oxidizing graphite powder referring to the modified Hummers method [36]. Typically, graphite powder (2 g), $NaNO_3$ (1 g), $KMnO_4$ (6 g) and concentrated H_2SO_4 (98%, 60 mL) were mixed and vigorously stirred in an ice bath for 2 h. Then, the resulting pasty and black-greenish mixture was placed in a 35 °C water bath and maintained at this temperature for 2 h, followed by the slow addition of distilled water (150 mL). Thereafter, the temperature was raised to 98 °C and the reaction was allowed to proceed at this temperature for 30 min. After the temperature decreased to 60 °C, H_2O_2 (30%, 10 mL) was then added and further stirred for anther 2 h. The solid product was filtered and thoroughly washed with hydrochloric acid (5%) and distilled water for several times until the pH of the washing water was neutral, and subsequently dried in a vacuum oven at 60 °C. Exfoliation of GO was carried out by sonication for 2 h in an aqueous solvent.

The magnetic graphene oxide (MGO) nanocomposites were prepared by co-precipitation method under alkaline solution over addition of ammonia solution [37]. Briefly, 500 mg of GO was dispersed in distilled water (200 mL) and then subjected to ultrasound for 3 h to form stable GO suspension. Meanwhile, $FeCl_3{\cdot}6H_2O$ (3 g) and $FeSO_4{\cdot}7H_2O$ (2.1 g) were dissolved in distilled water (100 mL) under the protection of nitrogen, with subsequent addition of ammonia solution under vigorous stirring at 80 °C. Afterward, the GO suspension was added into the above mixed solution with stirring, and then ultrasonicated for 45 min. Subsequently, the obtained MGO products were collected from the mixture by applying an external magnetic filed, washed with distilled water for several times, and finally dried in a vacuum oven at 60 °C for further utilization.