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Lipase oriented-immobilized on dendrimer-coated magnetic multi-walled carbon nanotubes toward catalyzing biodiesel production from waste vegetable oil

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HIGHLIGHTS

- The relationship of N content and magnetism of APTES modified m-MWCNTs was studied.
- RML was oriented-immobilized based on the 3D structure analysis of molecule.
- m-MWCNTs-PAMAM-lipase was easily recovered and recovery activity up to 2808%.
- Biodiesel yield reached 94% and had no significant decrease after 10 cycles.

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ABSTRACT

A polyamidoamine (PAMAM) dendrimer was grafted onto magnetic multi-walled carbon nanotubes (m-MWCNTs) to combine magnetic properties with a large surface functionalized with amino groups. Based on three dimensional structural (3D) analysis of enzyme, oriented-immobilization of *Rhizomucor miehei* lipase (RML) on the obtained m-MWCNTs-PAMAM matrix was achieved. The recovery activity of the immobilized lipase was up to 2808% and the corresponding esterification activity was 27-fold higher than that of the free enzyme. The immobilized enzyme was employed to catalyze biodiesel production from waste vegetable oil in a *tert*-butanol solvent system. Biodiesel conversion reached 94% under optimal conditions. Moreover, the immobilized lipase could be easily recovered and there was no significant decrease in conversion rates after 10 cycles of reuse. The results suggested that the immobilized RML is a potential catalyst with high stability and excellent operational reusability for biodiesel production.

1. Introduction

Biodiesel, as a renewable, environmentally friendly and biodegradable biofuel, can address the energy crisis and environmental pollution associated with the combustion of fossil fuels [1]. It can be produced by esterification or transesterification of edible and non-edible oils, where triglycerides react with a short-chain alcohol (ethanol or methanol) in the presence of chemical catalysts or enzymes [2]. The application of chemical methods has been limited, however, because of high energy requirements, difficulties in recovery of the catalyst and environmental pressure [3]. Recently, the enzyme (lipase)-catalyzed approach has received greater attentions since this bioprocess works under mild conditions and permits the use of raw materials containing large amounts of free fatty acids [2]. As yet, there are few industrialization plants using enzymatic biodiesel production technologies because of the high cost of feedstock and lipases. Therefore, the usage of non-edible oils such as *Jatropha curcas* seed oil [4], yeast oil [5], castor oil [6], microalgae oil [7] and waste oil [8] for biodiesel synthesis is more promising than the use of edible oils. Additionally, immobilization can effectively improve the stability and reusability of lipases [9].

Recently, various magnetic nanocomposites including nanoparticles, nanotubes and grapheme, have been extensively studied for the immobilization of lipases [10–12]. These materials minimize mass transfer resistance, simplify industrial waste treatment due to their small size, and allow easy recovery of the supported-enzyme conjugates by means of a simple magnet [13]. Among these nanomaterials, magnetic carbon nanotubes (m-CNTs) have become a hotspot of interest because of their large specific surface area, highly polar functional groups on the surface, a rich porous

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structure, good chemical stability and efficient heat conductivity [14]. Nevertheless, m-CNTs do not provide enough active sites to immobilize lipases. It has been demonstrated, however, that magnetic multi-walled carbon nanotubes functionalized with dendrimer molecules can provide massive active sites to increase the loading of glucoamylase [13]. Until now, there have been no reports on lipases immobilized onto dendrimer-coated magnetic multi-walled carbon nanotubes (m-MWCNTs-PAMAM).

Rhizomucor miehei lipase (RML), a 1,3-specific lipase [15], is usually used as catalyst in free, immobilized, and whole-cell forms for biodiesel preparation [7,16,17]. Thus, in this work, m-MWCNTs-PAMAM was prepared and used for oriented-immobilization of RML based on 3D structural analysis of the enzyme. Optimization of the immobilization conditions was also investigated. The conjugate of m-MWCNTs-PAMAM with lipase was utilized to catalyze transesterification of waste vegetable oil in a *tert*-butanol solvent system into biodiesel, and the reaction conditions were optimized to improve the conversion rate.

2. Materials and methods

2.1. Materials

R. miehei lipase (RML) was purchased from Sigma Aldrich (Shanghai, China) and used without further purification. MWCNTs (40–60 nm diameter, purity >95%) were from Nanotech Port Co., Ltd. (Shenzhen, China). Waste vegetable oil was obtained from ZTE Agri-valley Co., Ltd. (Hubei, China). It has a relatively high acid value of 11.6 mg KOH/g. The composition of the waste oil was presented in Table S1 and Fig. S1. Glutaraldehyde, a commonly used non-toxic cross-linker, (3-aminopropyl) triethoxysilane (APTES), N-ethyl-N-(3-(dimethylamino) propyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS)and other organic solvents of analytical grade were bought from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) without further purification.

2.2. Methods

2.2.1. Analysis of the 3D-structure of RML

Two 3D structural models of RML (pdb identifiers: 3TGL, closed configuration; 4TGL, open configuration) obtained from the NCBI (http://www.ncbi.nlm.nih.gov/) were employed to analyze surface-exposed amino acid groups using PyMOL (2.7.6) [18].

2.2.2. Synthesis of m-MWCNTs-PAMAM composites

2.2.2.1. Preparation of m-MWCNTs. The m-MWCNTs were prepared according to the method of Chen et al. [19] with a slight modification. The detailed preparation procedures are provided in the Supporting Information.

2.2.2.2. Surface modification with PAMAM dendrimer. Dendrimer growth was performed as described by Zhao et al. [13]. A brief outline is shown in the Supporting Information.

2.2.3. Lipase immobilization

Immobilization of the lipase by covalent bond formation between amine groups and carboxyl groups on the enzyme molecules is detailed in the Supporting Information.

The amount of immobilized enzyme was determined by measuring the protein content of the original lipase solution, and in the supernatants and washing solutions after immobilization, using the method of Bradford with bovine serum albumin (BSA) as the standard protein [20]. The immobilized enzyme was stored at 4 °C prior to use.

2.2.4. Enzyme assays

The esterification activities of the immobilized and free lipases were assayed using the method described previously [21]. A brief depiction is presented in the Supporting Information.

2.2.5. Measurement

The dimensions and morphological details of the composites were visualized by transmission electron microscopy (TEM, H-7000FA, Hitachi). TEM specimens were prepared by placing a drop of the sample suspension on a carbon-coated copper grid. The magnetic properties were measured using a superconducting quantum interference device (SQUID, Quantum Design) at room temperature. The structure of the particles was examined using an X'Pert PRO X-ray diffractometer with Cu radiation (PAN analytical B.V., Almelo, Netherlands). The FT-IR spectra were obtained in transmission mode on a Fourier-transform infrared spectroscopy (Bruker, VERTEX 70, Germany) using the KBr pellet technique. Nitrogen content in each generation of the m-MWCNTs-PAMAM nanocomposites was determined with a Vario Micro cube elemental analyzer (Elementar Co., Germany).

2.2.6. Enzyme catalyzed biodiesel production

Waste vegetable oil and short-chain alcohols (methanol or ethanol) were used as the substrates for biodiesel production in a *tert*-butanol organic solvent system, using the immobilized RML as biocatalyst. Reactions were conducted in a 50 mL stoppered shaking flask at a stirring rate of 200 rpm for 30 h under certain temperature. Firstly, 1.98 g waste vegetable oil was added to the reactor. Water was added dropwise and mixed with oil, followed by addition of the immobilized lipase (2.387×10^4 U/g). The respective alcohols were added in three steps at the same interval and a molar ratio of alcohols:oil of 4:1. All dosages were based on the oil weight, unless stated otherwise. The enzyme in the reaction mixture was recovered using a magnet and then added into the next batch to determine the reusability of the immobilized lipase.

The fatty acid alkyl esters (biodiesel) were quantified using a GC-9790 gas chromatograph (Agilent HP-INNOWAX capillary column 30 m × 0.25 mm × 0.25 μm, J&W Scientific, Folsom, CA, USA) (see Supporting Information). Fatty acids in the waste vegetable oil were analyzed by gas chromatography–mass spectrometry (GC–MS) and thin-layer chromatography (TLC). The GC–MS (7890A/5975C, Agilent) was equipped with an HP-INNOWAX capillary column (30 m × 50 μm × 0.25 μm), and the operating conditions were those described by Yan et al. [22]. TLC analysis was performed according to Guan et al. [23].

2.2.7. Statistical analysis

All experiments were conducted in three parallel replicates. The data were analyzed using SAS 9.0, and the graphs were plotted using Origin 8.0.

3. Results and discussion

3.1. Chemical properties of the waste vegetable oil

Waste vegetable oil was analyzed by GC–MS and TLC. GC–MS was conducted for qualitative and quantitative analysis of fatty acid profiles. TLC analysis was also conducted. The results were listed in Table S1 and Fig. S1 of the Supporting Information.

3.2. Structural analysis of RML

The 3D structure of RML [24] was analyzed to identify surface-exposed amino acid residues using PyMOL. As is known, functional groups on the surface of the enzyme, usually including amino,