Efficient Biodiesel Production Catalysed by Lipase Immobilized on Nanowire Coils

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Abstract. The lipase from Candida rugosa (CRL) was covalently immobilized on various carbon nanomaterials (functionalized nanowire coils and multi-walled carbon nanotubes) and tested for biodiesel production. Using the most active lipase preparation (covalently immobilized CRL on Nanowire coils) under optimal conditions, 96.8% conversion of waste oil was obtained after only 12 h reaction time. Moreover, the biocatalyst maintained nearly 90% of its initial activity in the batch system after 13 recycling experiments and still 80% initial activity after storage 240 d.

1. Introduction

The current society is highly dependent on fossil fuels and their extensive use resulted in continuously pollution and global warming-related problems. Biodiesel is a good substitute for fossil diesel due to its unique characteristics of renewability, biodegradability, nontoxicity, and clean combustion. Chemical-catalysed esterification or transesterification of oils and fats with short-chain alcohol is industrially adopted method for biodiesel production. However, the process expansion production and popularization are plagued by the harsh conditions, high energy consumption, corrosive nature of the catalyst, and so forth. Enzymatic synthesis of biodiesel is more economically and environmentally than the chemical method, but it also has some disadvantages such as high cost, poor stability and reusability. Immobilization technology is an efficient way to solve such problems. By covalent binding or embedding enzyme to support, the activity and stability of the enzyme can be improved effectively through changes in microenvironment and molecular conformation.

Mesoporous silica nanowire coils (NWCs) with three-dimensional (3D) networks are a class of fascinating functional materials and have attracted enormous research interest. The nanowire coils own multimodal pore system that beneficial to mass transport taking place in both reaction and sorption processes. For instance, shorter mesopores perpendicular to the axial direction of silica nanowire would allow more catalyst impregnation and larger effective surface areas for catalytic reactions and/or sorption processes. In addition to nominal surface area and porosity, orientation or architecture of the mesopores is another important property that can provide advantages for enzyme immobilization. Yet, as far as we know, no one has reported the application of silica nanowire coils in enzyme immobilization in biodiesel production.

Since lipase from Candida rugosa immobilized on various functional materials proved to be an efficient biocatalyst for biodiesel synthesis. Herein, the present study develops a highly active and stable biocatalyst for the conversion of waste oil with methanol into biodiesel through the covalent
immobilization of CRL on NWCs. The effect of the reaction parameters on the yield of biodiesel is optimized.

2. Materials and Methods

2.1. Materials

*Candida rugosa* lipase (16.7 mg protein/mL) and N435 were purchased from Beijing Cliscent Technology Co., Ltd. NH$_2$-functionalized multiwalled carbon nanotubes (MWCNTs-NH$_2$) were purchased from Chengdu Organic Chemicals Co. Ltd., China. Glutaraldehyde (GA), waste oil, tetraethyl orthosilicate (TEOS), triethanolamine (TEA) and hexadecyltrimethylammonium chloride (CTACl) were purchased from Tianjin Chemical Corporation (Tianjin, China). 3-amino-propyltriethoxysilane 98% (APTES) and divinylsulfone (DV) were purchased from Sigma Chemical Co (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade obtained from a local store.

2.2. Preparation of Nanowire Coils (NWCs)

Mesoporous silica nanowire coils were synthesized by hydrolysis of TEOS with TEA as an alkaline source. Firstly, deionized water (13 mL), methanol (1 mL), 25% CTACl aqueous solution (2 mL) and TEA (0.5 mL) was added into a pressure flask in order. The resultant mixture was stirred at room temperature for 30 min. Then, 0.5-1.5 mL of TEOS was dropwise added under magnetic stirring. Finally, the mixture was heated in drying oven at 70 °C for 1.5 h. The opaque precipitates (NWCs) were obtained by adding ethanol and centrifuged at 8000 rpm for 2 min.

2.3. Covalent Immobilization of CRL

Covalent Binding of CRL to NWCs. 0.2 g NWCs were mixed with APTES (0.6 mL), toluene (8 mL) and ethanol (4 mL) by sonication 3 times during 5 min at room temperature. Afterwards, the material was washed with methanol and ethanol to remove the excess of APTES. The resultant material (1g) was suspended in PBS buffer (20 mM, pH 7, 5 mL) containing 15% (v/v) of DV and then oscillated at room temperature for 30 min. Then, 0.5-1.5 mL of TEOS was dropwise added under magnetic stirring. Finally, the mixture was heated in drying oven at 70 °C for 1.5 h. The opaque precipitates (NWCs) were obtained by adding ethanol and centrifuged at 8000 rpm for 2 min.

Then, CRL@NWCs-DV was freeze-dried and used in subsequent enzymatic tests.

Covalent Binding of CRL to MWCNTs. MWCNTs-NH$_2$ (100 mg) was suspended in a PBS solution (5 mL, 10 mM, pH 7.0) containing 0.2% glutaraldehyde and was magnetically stirred for 1 h at 30 °C. The suspension was then centrifuged, and the solid was washed with distilled water three times. After activation, the material was dispersed into a phosphate buffered saline containing CRL (0.5 g) at 30 °C under stirring for 30 min. Then, the mixture was centrifuged and washed three times with buffer solution. The obtained solid (named CRL@MWCNTs-GA) was freeze-dried and stored at 4 °C until it was used.

2.4. Enzymatic Synthesis of Biodiesel

Enzymatic methanolysis reactions were performed using 40 mg oil (50 µmol) in 1 mL organic solvent, 6 mg catalyst and 13 µL methanol. The mixture was shaken (400 r/min) at room temperature for 8 h and then centrifuged at 10000 r/min for 1 min. The solvent was removed from the supernatant and analysed using gas chromatography (GC) to calculate the biodiesel yield.

GC analysis was performed Agilent 7890A GC equipped with an FID and SE-30 capillary column. Nitrogen gas flowrate was 20 mL/min. The sampler and the detector temperature were 230 °C and 285 °C, respectively. The initial temperature of column was 160°C and was heated to 220 °C at 15 °C/min 2 min later. Then, the temperature rose to 260 °C within 5 min and maintained for 10 min. The sample was prepared with the reaction mixture, n-hexane and salicylate acid methyl ester (volume ratio 1:1:2).
The effect of the preparation parameters, including the solvent (acetone, dimethylsulfoxide, methanol, cyclohexane, CCl₄, and t-butanol), water content, catalyst dosage, methanol: oil molar ratio and time was investigated in general procedure.

3. Results

3.1. The Effect of Organic Solvents upon Biodiesel Production

Figure 1 shows the performance of catalyst in the enzymatic production of biodiesel after incubated in different solvent for 4 h. The Log P rage of the selected solvents was -1.3 to 3.2, covering both polar and non-polar solvents. It is well known that polar solvents can snatch the necessary water in lipase, leading to conformational changes of molecules and decrease the activity. Therefore, CRL@MWCNTs-GA, CRL@NWCs-DV and CRL displayed poor or moderate performance after being pre-treated with acetone (-0.23), dimethylsulfoxide (-1.3) and methanol (-0.76), respectively. Conversely, the catalysts all owned a better activity after incubated in cyclohexane (3.2), CCl₄ (3.0) and t-butanol (0.8). This is attributed to the non-polar solvent can stabilize the lipase conformation and was conducive to open the “lid” in active site, promoting the binding of substrates to the active center[1]. In the reaction process, free CRL tended to agglomerate, leading to enhanced diffusion inhibition and inhibition of substrate transformation, which could be avoided in the immobilization process[2]. In addition, the hierarchical mesoporous structure in CRL@NWCs-DV and multi-site immobilization between CRL and NWCs by DVS both provided additional support and protection for CRL, effectively avoiding the destruction of molecular structure by the harsh environment, thus maintaining the CRL stable state. Consequently, CRL@NWCs-DV displayed the best performance in the experiments.

![Figure 1 The effect of organic solvents on catalysts in biodiesel production](image)

3.2. The Effect of Water Content on Biodiesel Production

The activity and the stability of biocatalysts, including the immobilized lipase, are strongly affected by the water content in the system. Therefore, the influence of water content from 0 to 5 wt.% on the performance of CRL@MWCNTs-GA and CRL@NWCs-DV was investigated. It can be known from Figure 2 that the optimal water amount for CRL@MWCNTs-GA and CRL@NWCs-DV was 1.0 % and 0.5%, respectively. Once deviating from the optimal dosage, the activity of both catalysts would decrease. This was because the lipase required certain amount of water to form a hydration layer and maintained the spatial conformation of the enzyme protein. However, excess water in the reaction mixture would inhibit the esterification reducing the conversion. The reason why the optimal water content of CRL@NWCs-DV was lower than that of CRL@MWCNTs-GA might be that the multistage mesoporous structure of CRL@NWCs-DV could enrich the water molecules in the system, which was conducive to the formation of hydration layer[3]. However, CRL@MWCNTs-GA lacked adsorption capacity and had a low utilization rate of the water in the system, so more water needed to maintain its conformational mobility.
3.3. The Effect of the Methanol to Oil Molar Ratio on Biodiesel Production

Although increasing the amount of methanol can improve the oil conversion rate, but the high concentration of methanol will destroy the molecular structure, inhibiting the lipase activity and reducing the reaction rate[4]. Based on this phenomenon, we studied the influence of molar ratio of alcohol to oil (constant amount of oil) on biodiesel production and selected the optimal ratio with the least harm to lipase and the maximum conversion. Figure 3 showed that when the molar ratio of alcohol to oil was 5:1, the oil conversion was the highest and any deviation from this molar ratio would lead to a decrease.

3.4. The Influence of Catalyst Dosage on Biodiesel Production

Figure 4a revealed the effect of catalyst dosage on biodiesel production. Consistent with other reports[5], the substrate conversion rate was increasing with the increase of catalyst (CRL@MWCNTs-GA and CRL@NWCs-DV) dosage, but the increase rate decreased. When the addition amount of CRL@MWCNTs-GA further increased from 10 to 15 wt.% and CRL@NWCs-DV increased from 7 to 15 wt.%, the conversion did not change significantly. The lower CRL@NWCs-DV dosage indicated that its performance was superior to that of CRL@MWCNTs-GA, which may be attributed to the hierarchical mesoporous structure of NWCs.

Figure 4b showed that, the conversion gradually increases with the extension of reaction time. In CRL@NWCs-DV catalytic systems, the conversion rate reached a maximum of (very close to) 96.8% at 12 h. While in the CRL@MWCNTs-GA system, the conversion was only 94.3% after 12 h, which was close to the maximum value of 95.6% (14 h). As the reaction time continued to increase, the conversion of the two systems changes slightly, and the input was not proportional to the output.
3.5. **Storage Stability and Reusability of the Immobilized Lipases**

As shown in Figure 5a, the residual activity of CRL@NWCs-DV and CRL@MWCNTs-GA still retained about 80% of the initial activity after 240 days, while the free enzyme only retained 17% of the initial activity. The results showed that immobilization could avoid the aggregation of lipase molecules, which occurred during the storage of CRL, thus effectively prolonging the storage time of the catalyst[2]. In addition, the solid connection between CRL and NWCs or MWCNTs enhanced the rigidity of the lipase structure, thus improving the stability of CRL@NWCs-DV and CRL@MWCNTs-GA[6].

Figure 5b recorded the times of repeated use of CRL@MWCNTs-GA and CRL@NWCs-DV and compared it with the equal activity catalysts (free CRL and N435). Obviously, free CRL had the least reusability while CRL@NWCs-DV had the best reusability. For CRL@NWCs-DV, the residual activity was 90% after 13 consecutive uses, which was higher than that of CRL@MWCNTs-GA (84%) and N435 (30%). This was because support could provide protection for the lipase and reduce the damage to the structure from the adverse environment in the continuous reaction. In the N435 catalytic system, there are less and less effective catalysts due to the desorption of lipase from resin leading to the decrease of calculated residual activity. For CRL@MWCNTs-GA and CRL@NWCs-DV, the lipase was covalently linked to the carrier, making it more difficult to break off and thus continue to play catalytic role[7]. In CRL@NWCs-DV, more binding sites attributed to DV between the lipase and the NWCs made its structure more stable[8].

4. **Conclusions**

In this study, *Candida rugosa* lipase was covalently bonded to functionalized nanowire coils and multi-walled carbon nanotubes and the resulted catalysts were employed in biodiesel production. The enzymatic preparation providing a better performance was achieved when functionalized nanowire coils as support and DV as crosslinker were used (CRL@NWCs-DV). Under the optimal conditions of the batch procedure, the highest conversion was 96.8% catalysed by CRL@NWCs-DV. In the stability
test, CRL@NWCs-DV maintained nearly 90% of its initial activity in the batch system after 13 reused and still 80% initial activity after storage 240 d, showing better stability than CRL@MWCNTs-GA, N435 and CRL.

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