sustainability

MDPI

Article

Production of Biodiesel Using Immobilized Lipase and the Characterization of Different Co-Immobilizing Agents and Immobilization Methods

Kang Zhao [1,2], Qinjian Di [1], Xi Cao [1], Meng Wang [1], Li Deng [1,3,*] and Fang Wang [1,4]

1 Beijing Bioprocess Key Laboratory, College of Life Science and Technology, Beijing University of Chemical Technology, Beijing 100029, China; Zhaokang0109@163.com (K.Z.); dqj0209@163.com (Q.D.); caoxiex@163.com (X.C.); wang_huj@163.com (M.W.); wangfang@mail.buct.edu.cn (F.W.)
2 China National Publications Import & Export (Group) Corporation, Beijing 100020, China
3 Amoy—BUCT Industrial Bio-technovation Institute, Amoy 361022, China
4 State Key Laboratory of Chemical Resource Engineering, Beijing University of Chemical Technology, Beijing 100029, China
* Correspondence: dengli@mail.buct.edu.cn; Tel.: +86-10-6441-4543

Academic Editor: Michael Wachendorf
Received: 2 June 2016; Accepted: 1 August 2016; Published: 24 August 2016

Abstract: Lipase from *Candida* sp. 99–125 is widely employed to catalyzed transesterification and can be used for biodiesel production. In this study, the lipase was immobilized by combined adsorption and entrapment to catalyze biodiesel production from waste cooking oil (WCO) via transesterification, and investigating co-immobilizing agents as additives according to the enzyme activity. The addition of the mixed co-immobilizing agents has positive effects on the activities of the immobilized lipase. Three different immobilizing methods were compared by the conversion ratio of biodiesel and structured by Atom Force Microscopy (AFM) and Scanning Electron Microscopy (SEM), respectively. It was found that entrapment followed by adsorption was the best method. The effect of the co-immobilizing agent amount, lipase dosage, water content, and reuse ability of the immobilized lipase was investigated. By comparison with previous research, this immobilized lipase showed good reuse ability: the conversion ratio excesses 70% after 10 subsequent reactions, in particular, was better than Novozym435 and TLIM on waste cooking oil for one unit of lipase.

Keywords: immobilization; lipase; co-immobilizing agent; biodiesel; biocatalyst

1. Introduction

Biodiesel, defined as monoalkyl esters of long chain fatty acids, is environmentally friendly and shows great potential as an alternative liquid fuel [1,2]. It is usually produced by transesterification of plant oils or animal fats with chemical catalysts or lipase. As biodiesel is promising as a renewable source of fuel, the technology of its production has been developing rapidly during the past 20 years [2,3]. So, lipase catalyzed biodiesel production is currently a very interesting topic as it allows the use of rather mild reaction conditions which is eco-friendly [4,5].

However, there are two common drawbacks for industrial application with the use of lipase processes. One is the high cost of the biocatalyst. The other is that the lipase lacks stability during the processing and is difficult to recycle from the reaction mixture. To solve these problems, the method of immobilization has been used to improve lipase stability for repeated utilization. Among all the immobilization techniques available, adsorption as a simple and less expensive method in which high catalytic activity can be retained has a higher commercial prospect [6,7]. Generally speaking, the interaction in adsorption is not very strong, and some of the adsorbed enzyme will be desorbed

during operation. Whereas entrapment can be defined as physical restriction of enzyme within a confined space or network [8]. The lipase immobilized by entrapment is much more stable than physically adsorbed lipase. However, the problems such as leakage of the enzymes during continuous use and insufficient substrate-enzyme interactions still have to be faced [9].

Thus, immobilized lipase combined with adsorption and entrapment could protect the lipase inside the pores and avoid desorption. In such processes, the additives such as ionic liquids, polyols and surfactants have a positive function [10–12]. Zhang et al. [13] have compared the effect of almost one hundred varieties of co-immobilizing agents on the esterification, including surfactants, fatty acids, fatty alcohol, metal ions and others. They found that different agents' impacts on the activity of immobilized lipase varied, and co-immobilizing agents such as coconut oil, Tween80 and $MgSO_4$ had an obvious synergy on the enzyme catalysis. However, few works have been completed regarding co-additives on immobilized lipase's activity. In this study, five additives were added as co-immobilizing agents in the immobilization process. The concentration of co-immobilizing additive was optimized. Three different immobilized methods were compared on the basis of conversion ratio.

2. Materials and Methods

2.1. Materials

Lipase from *Candida* sp. 99–125 was made in our laboratory and the activity of the lipase power was 30,000 U/g [14]. WCO was obtained from Lvming Co. Ltd., Shanghai, China. Tween80 was purchased from Tianjin Fuchen chemical reagents factory, Tianjin, China. Coconut oil, PEG6000 and magnesium sulfate were purchased from Beijing Chemical Reagents Company, Beijing, China. Moreover, lecithin was purchased from Aoboxing Biochemical Technology Co. Ltd., Beijing, China. All other chemicals were of analytical grade and used without further purification. Deionized water was used throughout the experiments.

2.2. Immobilization Procedures

2.2.1. Adsorption Process (Immobilization by Adsorption)

Sodium phosphate (150 mM, 30 mL) buffer solution containing 1 g lipase was mixed with diatomite in a 100 mL conical flask. The mixture was well commingled by reciprocal shaker under the optimized condition. Different additives acting as co-immobilizing agents (including coconut oil, PEG6000, magnesium sulfate, Tween80 and lecithin) were investigated in the adsorption process as parameters. The lipase solution (with or without co-immobilizing agents) after adsorption was collected by filtration. Immobilized lipase, merely treated by adsorption, (adsorption immobilization, Catalyst A) was dried in air at room temperature and its activity was inspected.

2.2.2. Entrapment Process (Immobilization by Entrapment)

Lipase was mixed with sodium alginate solution, and then dripped into calcium chloride solution, directly forming Ca-alginate beads (entrapment immobilization, Catalyst B).

2.2.3. Adsorption Followed by Entrapment

The lipase solution (in Section 2.2.1) was mixed with 30 mL 1.5% (w/v) of sodium alginate solution. The mixture was then stirred thoroughly to ensure complete mixing. The mixed solution was dripped into 40 mM, 150 mL calcium chloride solution with a syringe; Ca-alginate beads were formed afterwards. The beads were separated from the $CaCl_2$ solution by vacuum filtration after 1 h of hardening, air-dried at room temperature. The ultimately-immobilized lipase (adsorption-entrapment immobilization, Catalyst C) was obtained.

2.3. Measurement of Immobilized Lipase Activity

The hydrolytic activity assay was measured by titrimetric assay according to an olive oil emulsion method with some modifications [15]. Olive oil was emulsified in 2% (*w*/*v*) polyvinyl alcohol (PVA), the proportion of olive oil and 2% PVA is 1:3, in a homogenizer for 6 min at maximum speed. Then the lipase solution (1 mL, pure or diluted, depending on the quantity of lipase), was added into the mixture of substrate emulsion (5 mL) and phosphate buffer (4 mL, 200 mM, pH 8.0). Samples were incubated for 10 min at 40 °C. The reaction was stopped by adding 20 mL ethanol afterwards. Lipase activity was determined by titration of the fatty acid released with 50 mM NaOH. One activity unit of lipase was defined by 1 μmol of fatty acid released per minute under assay conditions.

2.4. Biodiesel Production from Waste Cooking Oil

Typical transesterification of WCO was carried out in a 100 mL conical flask, incubated in a reciprocal shaker at 40 °C and 180 rpm. The reaction system contained 20 g WCO, different contents of distilled water, immobilized lipase and 6 subsequent addition of 483 μL methanol, with a total reaction time of 24 H. Every equivalent of 483 μL methanol was added to the system every 3 H for six times. At a pre-determined time, 10 μL of the samples was taken and dissolved in 1.5 mL n-hexane for gas chromatography analysis. All the experiments were replicated at least three times and the results presented are the average values for the replicated data [16].

2.5. Analytical Procedure

Fatty acid methyl esters (FAME) yields were quantified by gas chromatography (GC). GC analysis used Thermo-TRACE1300 equipped with DB-1ht capillary column (30.0 m × 0.25 mm × 0.1 μm; J&W Scientific Columns, Agilent Technologies, Palo Alto, FL, USA) and a flame ionizing detector (FID). The column temperature was set at 100 °C, firstly heated by a speed of 15 °C/min to 180 °C, maintained for 5 min, and secondly heated by a rate of 20 °C/min to 350 °C, maintained for another 15 min. Nitrogen was used as the carrier gas and the injector and detector temperatures were both at 360 °C.

2.6. Operational Stability of Immobilized Lipase

In order to evaluate the stability of immobilized lipase in repeated use, a batch of transesterifications of WCO and methanol were conducted by the addition of immobilized lipase. After separation, the recycled immobilized lipase was reused with fresh substrates for each cycle. The reaction conditions were the same as described in Section 2.4.

2.7. Characterization with Scanning Electron Microscopy (SEM) and Atom Force Microscopy (AFM)

The structure of immobilized lipase was investigated using scanning electron microscopy (SEM) (HITACHI SU1510). All samples were sputter-coated with platinum prior to observation.

To better understand the free lipase structure and dimension, a nanoscope VIII MultiMode atomic force microscope (Digital Instruments, Santa Barbara, CA, USA) was employed in ScanAsyst mode. The sample was prepared for AFM by dropping 10 μL of lipase solution on a freshly cleaved mica surface for 10 s. The mica surface was rinsed extensively by water and dried gently with nitrogen gas. Topographic images and peak force error images were concurrently recorded under ambient conditions at 512 × 512 pixel resolution and a scanning speed of 1 Hz.

3. Results and Discussion

3.1. Effects of Co-Immobilized Agent in the Immobilized Process

3.1.1. Effects of *Co-Immobilized Agent* in the *Adsorption Process* of Immobilization

The use of additives has been reported as method to increase the catalytic activity of the lipase. Generally speaking, additives were including in surfactant, protein and salt molecules [12]. Additives

such as Tween80 and lecithin can protect the enzyme activity through contact with the enzyme active sites [17,18]. Based on these studies, a series of experiments were conducted with different additives, such as coconut oil, PEG6000, $MgSO_4$, Tween80 and lecithin. The optimized quantities of selected additives were shown in Figure 1a–e, which were 0.004 g, 0.008 g, 0.004 g, 0.008 g and 0.004 g, respectively. Then the five additives were used collectively as mixed co-immobilizing agents. The test result of the co-immobilizing agents was shown in Figure 1f. The hydrolytic activity was 2.77 times of that of free lipase and 1.72 times of that of the one without additive added and 1.2 times that of coconut oil which showed the best hydrolytic activity.

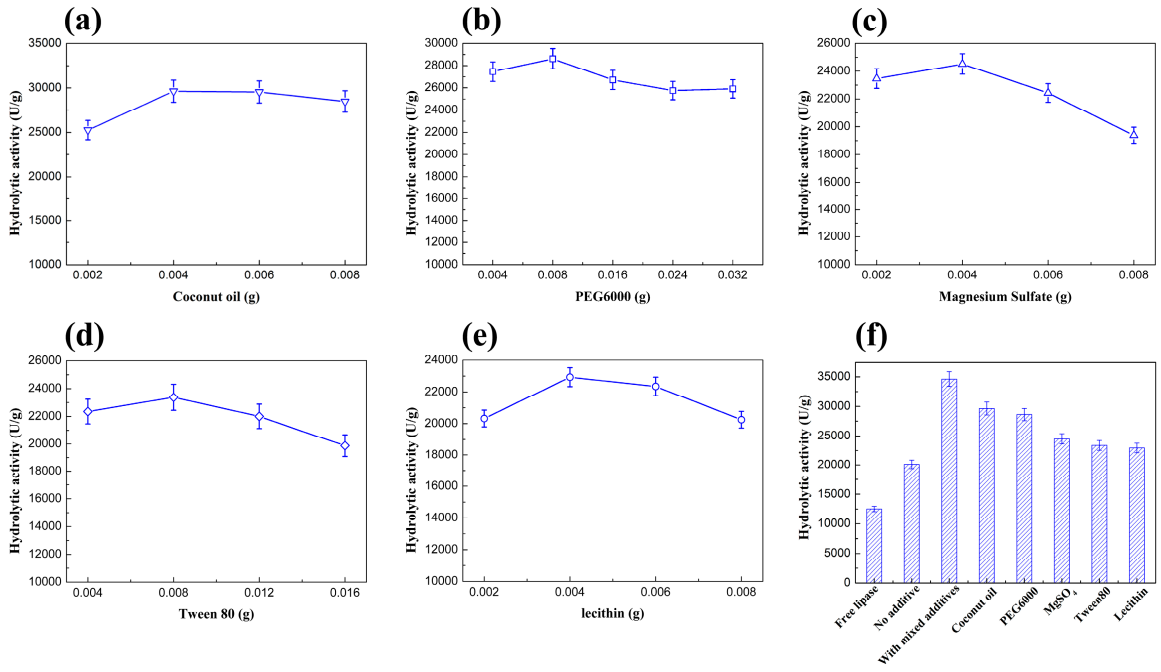

Figure 1. Effects of different additives on the hydrolyzing activities of immobilized lipase by adsorption (**a**) Coconut oil, (**b**) PEG6000, (**c**) $MgSO_4$, (**d**) Tween 80, (**e**) Lecithin and (**f**) Summary results of mixed additives, different single additives, no additives with immobilized lipase obtained only by adsorption and with free lipase.

3.1.2. The Optimized Concentration of Co-Immobilizing Agents

The lipase was treated with co-immobilizing agents in the adsorption process, and then followed by entrapment in alginate beads to obtain the ultimately immobilized lipase with the condition mentioned above. Because the biodiesel conversion was closely related with the concentration of the mixed co-immobilizing agents, the optimized concentration of co-immobilizing agents by comparing biodiesel conversion were shown in Figure 2. It indicated that conversions with co-immobilized agent concentration of 0.5% (*w*/*w*) and 1.5% were rapidly decreased after three cycles in the biodiesel reaction. However, the conversion of concentration with 1.0% co-immobilized agent was relatively stable and still nearly 80% after six recycles. Thus, the relatively optimum concentration level of co-immobilizing agents was 1.0%.

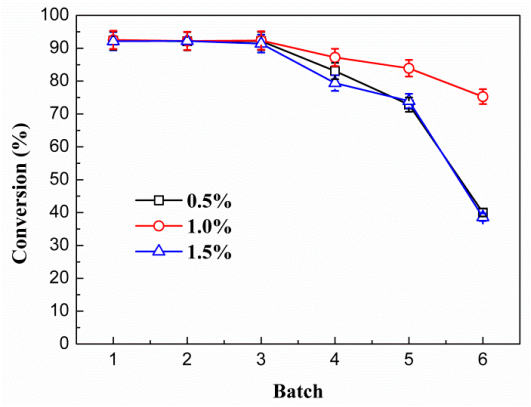

Figure 2. The optimization concentration of mixed co-immobilizing agents for the biodiesel reaction batches.

3.2. Biodiesel Production Using Immobilized Lipase as Biocatalyst

The immobilized lipase treated with mixed co-immobilizing agents as a biocatalyst was used to catalyze transesterification of WCO with methanol to produce biodiesel. The main reaction conditions were optimized by adjusting the amount of immobilized lipase and the water content. In order to find the optimal amount of the immobilized lipase, the effects of different amount of immobilized lipase on the transesterification rate of the WCO and the results of repeated use of immobilized lipase were compared. Three biocatalysts obtained by different immobilized methods were compared in the biodiesel reaction (see Section 2.2, catalysts A, B and C).

3.2.1. The Amount of Immobilized Lipase

As shown in Figure 3a, the conversion ratio was improved obviously by adding 0.6~1.2 g immobilized enzyme with its marginal increase peaked at the point that 0.8 g immobilized enzyme was added to the reaction when the ratio turned to be 91.55%. Afterwards the conversion ratio did not show any significant enhancement when adding more biocatalyst. This can be explained as follows: if the amount of substrates was fixed, the reaction rate was determined by the number of active sites provided by the combined lipases [19]. When the amount of substrates was higher than the number of active sites, adding more biocatalyst to the reaction system may result in an obvious increase in conversion. However, when the immobilized lipase dosage was over 0.8 g, no augmented conversion was observed due to the limited quantity of substrate molecules.

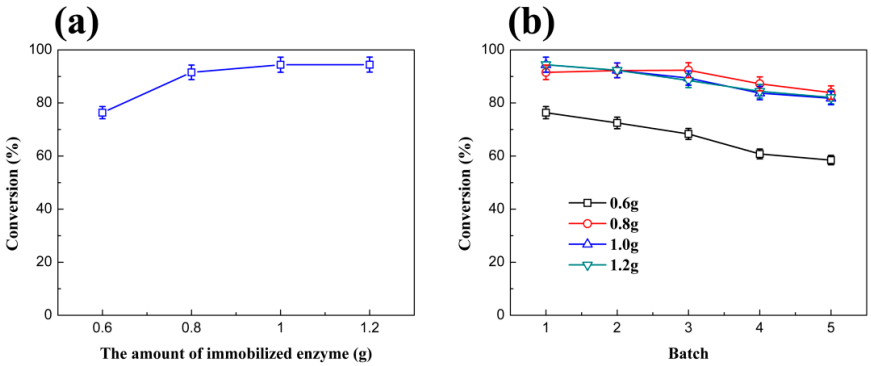

Figure 3. (**a**) The influence of the amount of immobilized enzyme for biodiesel conversion; and (**b**) the influence of the amount of immobilized enzyme for biodiesel reaction batches.

Whether the immobilized lipase can be reused or not is another main issue. The reuse results with different amounts of immobilized lipases were shown in Figure 3b. It was obvious that the biodiesel conversion ratio still remained 80% after 5 cycles with the immobilized lipase addition of 0.8, 1.0 and 1.2 g. So the 0.8 g immobilized lipase was chosen as the best dosage for economy.

3.2.2. The *A*mount of the Water Content

One of the distinct characteristics of this lipase was that it needed much more water to maintain a high transesterification activity. Therefore, water may take part in the transesterification and had an influence on the reaction equilibrium [20,21]. To find the most suitable amount of water content, the effects of different amounts of water on conversion ratio and the results of reusing of immobilized lipase were compared. The reactions were performed taking water content ranging from 1% to 6% (w/w) of the total amount of reaction mixture with the rest of the conditions unchanged.

As shown in Figure 4a, the conversion ratio with different water content had no significant change. However, in the batches reactions as shown in Figure 4b, water content had a big impact on reusing conversion. The activity of reused enzyme fell off rapidly under the low water content environment. After five consecutive reactions with the same lipase, the conversion of the biodiesel improved gradually with the increasing of water content. This phenomenon indicated that the lipase's active center can be protected by water. When the water content was increased to 5%, the lipase was fully protected, thus its activity cannot be further promoted with the continuously increasing of water content. Moreover, excess water may have some side-effects on the enzyme activity because it might make the lipase more flexible and may also lead to some unintended side-reactions such as hydrolysis, especially in the transesterification process [22]. Hence, 5% water content was considered to be the best condition in the later experiment.

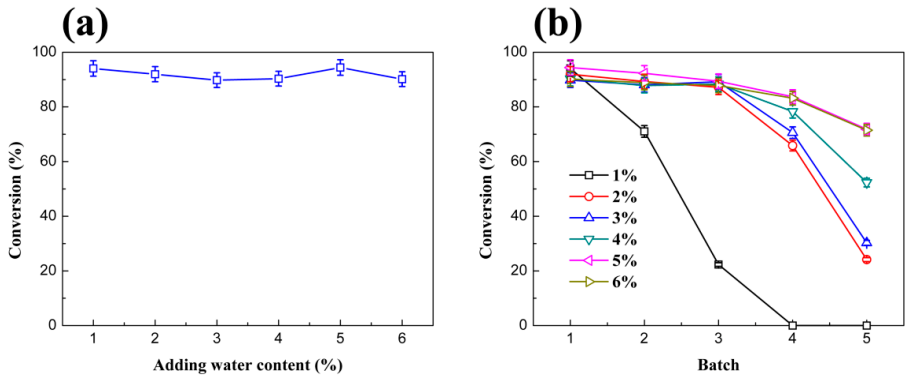

Figure 4. (**a**) The influence of water content on biodiesel conversion; and (**b**) the influence of water content on biodiesel reaction batches.

3.2.3. The Comparison of Different Immobilized Methods

Obviously, selecting a proper technique to immobilized lipase was very important. Lipase immobilization was carried out by ways of simple adsorption (catalyst A), entrapment (catalyst B) and entrapment followed by adsorption (catalyst C). The effects of different methods on immobilized lipase were evaluated by conversion ratio (Figure 5). The catalyst C had the best performance, while the catalyst B had the worst.

This happened because when using the entrapment technique, the network collapsed with the lack of interior support (diatomite) after free lipase entrapping into the alginate gel bead which then had no gap structure that can provide contact area for internal lipase and external substrate. That resulted in the conversion rate by catalyst B showed 28%, even lower than that of free lipase. If the techniques of adsorption and entrapment were combined, the lipase inside the pores would be well protected from desorption. So, catalyst C, with a conversion rate of 92%, had better stabilizations.

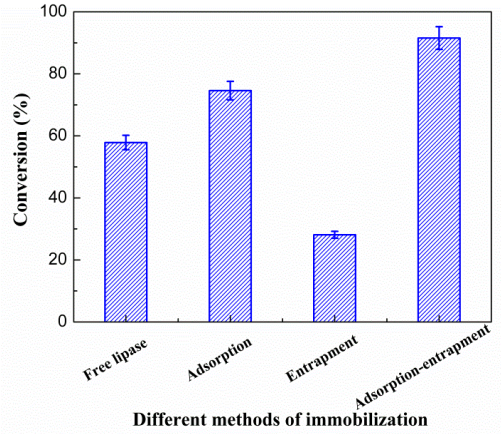

Figure 5. Comparison of different immobilized methods by biodiesel conversion ratio (free lipase, adsorption (Catalyst A), entrapment (Catalyst B) and adsorption-entrapment (Catalyst C)).

In order to better exhibit the difference between three immobilized methods (catalysts), the structure of free lipase and three immobilized lipase were examined by AFM [23] and SEM. The images of free lipase scanned by AFM were shown in Figure 6(A1–A3). The height image showed the presence of particles with the height of 5 nm (Figure 6(A1,A3)) [24]. The peak force error images (Figure 6(A2)) corresponded to the height image.

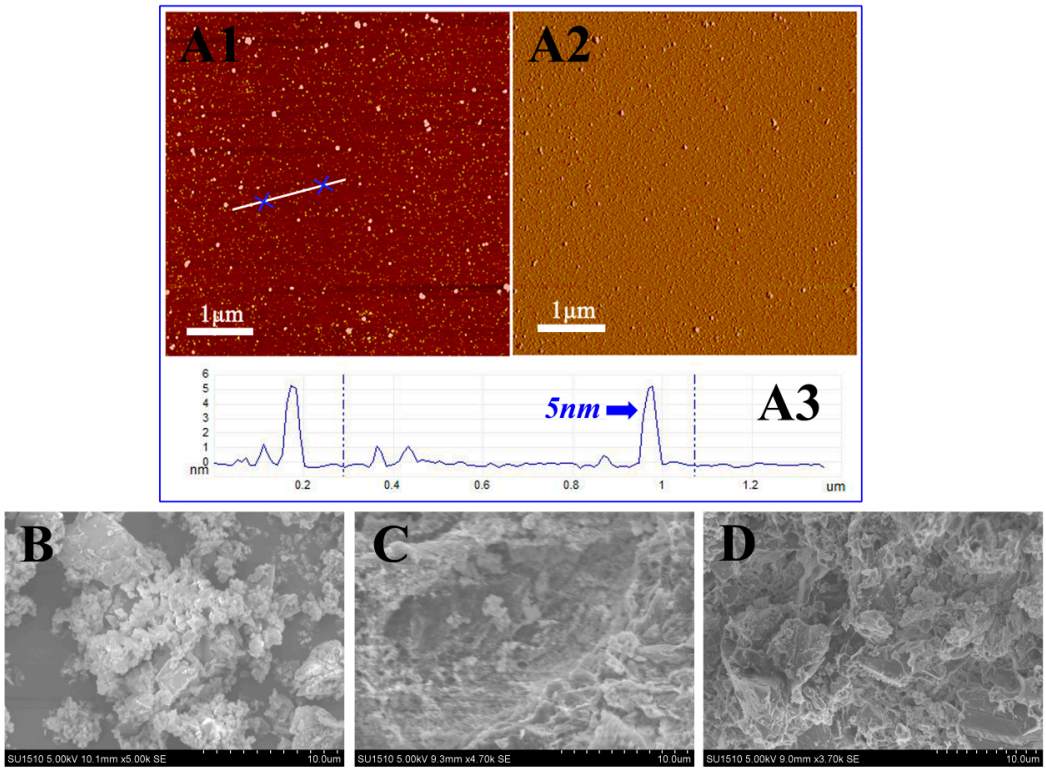

Figure 6. (**A1**) Typical Atom Force Microscopy (AFM) topographic images of free lipase; (**A2**) peak force error and (**A3**) corresponding height profiles of lipase. The Scanning Electron Microscopy (SEM) of (**B**) Lipase which was merely treated by adsorption onto diatomite (Catalyst A); (**C**) Lipase was entrapment into Ca-alginate gel (Catalyst B) and (**D**) the adsorption-entrapment immobilized lipase (Catalyst C).

Due to the amorphous morphology of both of lipase and diatomite, AFM results cannot show the interactions between lipase and diatomite during the adsorption process. However, SEM can well exhibit the morphology of the adsorption immobilization. The analysis results of lipase treated by adsorption, by entrapment and by combined techniques were shown in Figure 6B–D, respectively.

Diatomite-lipase clusters were found in Figure 6B; however, the formations of these aggregates (catalyst A) were electrostatic interaction rather than chemical forces. That means their interaction was not strong enough to prevent the enzyme from desorbing during the operation. Since the lipase was restricted within a confined space, the lipase immobilized by entrapment was very likely much more stable than that immobilized by adsorbing. However, as shown in Figure 6C, the cross section formed little network structure and were relatively compact, with a lack of interior support. So, it showed the worst conversion rate in the previous experiment (Figure 5). When lipase was immobilized by combined techniques, more porosity was found (shown in Figure 6D). Lipase and substrate can fully contact each other through the pores to obtain a high catalytically activity which was proved by the data shown in Figure 5.

3.3. The Operational Stability of Immobilized Enzyme

The reusability of the immobilized lipase is the most appropriate way to evaluate the immobilization procedure. Experiments were performed to examine the recyclability and the stability of the immobilized lipase. After each transesterification reaction, the immobilized lipase was recovered by filtration and subsequently reused.

The effect of biodiesel conversion was assayed for six cycles with or without co-immobilization agent in order to find out the difference between them. The immobilized lipase with co-immobilizing agent was able to maintain a good activity with over 80% conversion after six subsequent uses and exceeds 70% after 10 subsequent reactions. However, without co-immobilized agent, the conversion was below or near to zero after six cycles (Figure 7). The co-immobilizing agent has positive impacts on the biodiesel yields, because the batch biodiesel conversion was significantly higher than the one in which co-immobilized agent was not used.

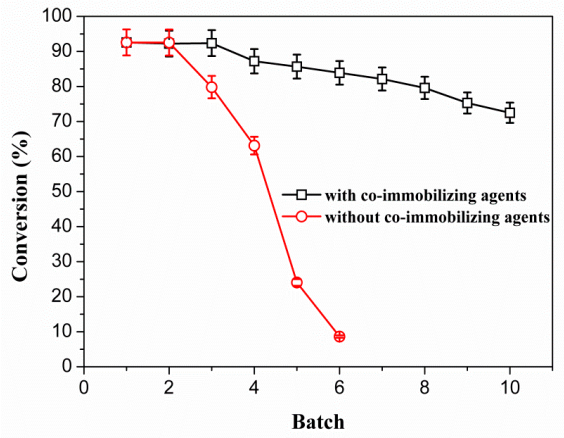

Figure 7. The operational stability of immobilized lipase.

For comparison, the results regarding immobilized lipase in this study and previous researches are shown in Table 1 [16,25–28]. In this work, the maximum biodiesel yield was obtained at 92% and the ultimate yield was over 70% after 10 times repeated experiments with the amount of lipase of 1%. The immobilized lipase with co-immobilizing agents had a good performance like other biocatalysts, and was obviously more durable than Novozym435 and TLIM in trasesterificating waste cooking oil by comparing each percentage of lipase used.

Table 1. Comparison of different immobilized lipases.

Lipase	Carried Used	Other Details	Oil	Percentage of Lipase Used (*w*/*w* of Oil)	Temperature (°C)	Maximum Yield (%)	Duration (h)	Batch	Ultimate Yield (%)	Reference
Candida sp. 99–125	Diatomite	With co-immobilizing agents	Waste cooking oil	1	40	>90	24	10	>70	This work
Candida sp. 99–125	Textile	Immobilized lipase immersed in organic solvents and salts	Soybean oil	10	40	>80	12	9	>50	[16]
Candida sp. 99–125	Textile	Textile pre-soaked with co-immobilization solution	Lard	20	40	>80	30	7	>70	[25]
Candida sp. 99–125	Cotton membrane	Membrane stirred with solutions containing gelatin, Tween-80, and PEG6000	Vegetable oil	15	40	>90	30	6	>90	[26]
Combined Novozym435 and TLIM	Acrylic resin and Silica gel	In tert-butanol medium	Waste cooking oil	4	50	>80	10	30	>70	[27]
Combined Novozym435 and TLIM	Acrylic resin and Silica gel	In tert-butanol medium	Lard	4	50	>90	20	20	>90	[28]

4. Conclusions

Selection of suitable immobilization technique was important to ensure effective usage of enzyme without leakage or detachment from the support. Co-additives can be better than single ones at enhancing immobilized lipase's activity. Three different immobilization methods were compared by the conversion ratio of the biodiesel from transesterification of waste cooking oil. The method of physical adsorption followed by entrapment to immobilize lipase had been proven to be a useful technique. Furthermore, the structure images by AFM and SEM also supported this theory. At the optimal conditions, the conversion rate was above 70% after 10 batches of reactions. The immobilized lipase had a good performance.

Acknowledgments: This research was financially supported by the National Key research programe (2016YFD0400601), Hong Kong, Macao and Taiwan scientific and technological cooperation projects (2015DFT30050), the Innovation Fund for Technology Based Firms (No. 14C26213511838), Amoy Industrial Biotechnology R&D and Pilot Conversion Platform (3502Z20121009), the Fundamental Research Funds for the Central Universities (ZY1522), BUCT Fund for Disciplines Construction and Development (No. XK1537).

Author Contributions: Kang Zhao participated in the design of the study, organized and interpreted the data, contributed to the critical discussion and drafted the manuscript. Qinjian Di and Xi Cao carried out the experiment and contributed to the data interpretation. Meng Wang coordinated part of the study and critically interpreted the data. Fang Wang contributed to the manuscript draft discussion and revised the manuscript. Li Deng coordinated the project, contributed to the critical discussion and data interpretation, and revised and corrected the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Demirbas, A. Political, economic and environmental impacts of biofuels: A review. *Appl. Energy* **2009**, *86*, S108–S117.
2. Narwal, S.K.; Gupta, R. Biodiesel production by transesterification using immobilized lipase. *Biotechnol. Lett.* **2013**, *35*, 479–490. [CrossRef]
3. Gog, A.; Roman, M.; Toşa, M.; Paizs, C.; Irimie, F.D. Biodiesel production using enzymatic transesterification–current state and perspectives. *Renew. Energy* **2012**, *39*, 10–16. [CrossRef]
4. Xie, W.; Wang, J. Immobilized lipase on magnetic chitosan microspheres for transesterification of soybean oil. *Biomass Bioenergy* **2012**, *36*, 373–380. [CrossRef]
5. Bajaj, A.; Lohan, P.; Jha, P.N.; Mehrotra, R. Biodiesel production through lipase catalyzed transesterification: An overview. *J. Mol. Catal. B Enzym.* **2010**, *62*, 9–14. [CrossRef]
6. Sun, J.; Jiang, Y.; Zhou, L.; Gao, J. Immobilization of candida antarctica lipase b by adsorption in organic medium. *New Biotechnol.* **2010**, *27*, 53–58. [CrossRef] [PubMed]
7. Gao, J.; Shi, L.; Jiang, Y.; Zhou, L.; He, Y. Formation of lipase candida sp. 99–125 cleas in mesoporous silica: Characterization and catalytic properties. *Catal. Sci. Technol.* **2013**, *3*, 3353–3359. [CrossRef]
8. Ertan, F.; Yagar, H.; Balkan, B. Optimization of α-amylase immobilization in calcium alginate beads. *Prep. Biochem. Biotechnol.* **2007**, *37*, 195–204. [CrossRef] [PubMed]
9. Wu, J.C.; Selvam, V.; Hui, H.T.; Chow, Y.; Talukder, M.M.R.; Choi, W.J. Immobilization of candida rugosa lipase by cross-linking with glutaraldehyde followed by entrapment in alginate beads. *Biocatal. Biotransform.* **2009**, *24*, 352–357.
10. Akoz, E.; Sayin, S.; Kaplan, S.; Yilmaz, M. Improvement of catalytic activity of lipase in the presence of calix arene valeric acid or hydrazine derivative. *Bioprocess Biosyst. Eng.* **2015**, *38*, 595–604. [CrossRef] [PubMed]
11. Cabrera-Padilla, R.Y.; Melo, E.B.; Pereira, M.M.; Figueiredo, R.T.; Fricks, A.T.; Franceschi, E.; Lima, Á.S.; Silva, D.P.; Soares, C.M. Use of ionic liquids as additives for the immobilization of lipase from bacillus sp. *J. Chem. Technol. Biotechnol.* **2015**, *90*, 1308–1316. [CrossRef]
12. Li, W.; Chen, B.; Tan, T. Comparative study of the properties of lipase immobilized on nonwoven fabric membranes by six methods. *Process Biochem.* **2011**, *46*, 1358–1365. [CrossRef]
13. Zhang, J.; Xu, J. Oleyl oleate synthesis by immobilized lipase from candida sp. 1619. *Chin. J. Biotechnol.* **1995**, *11*, 325–331.
14. He, X.-L.; Chen, B.-Q.; Tan, T.-W. Enzymatic synthesis of 2-ethylhexyl esters of fatty acids by immobilized lipase from candida sp. 99–125. *J. Mol. Catal. B Enzym.* **2002**, *18*, 333–339. [CrossRef]

15. Yu, M.; Qin, S.; Tan, T. Purification and characterization of the extracellular lipase lip2 from yarrowia lipolytica. *Process Biochem.* **2007**, *42*, 384–391. [CrossRef]
16. Lu, J.; Nie, K.; Xie, F.; Wang, F.; Tan, T. Enzymatic synthesis of fatty acid methyl esters from lard with immobilized candida sp. 99–125. *Process Biochem.* **2007**, *42*, 1367–1370. [CrossRef]
17. Secundo, F.; Carrea, G. Mono- and disaccharides enhance the activity and enantioselectivity of burkholderia cepacia lipase in organic solvent but do not significantly affect its conformation. *Biotechnol. Bioeng.* **2005**, *92*, 438–446. [CrossRef] [PubMed]
18. Rathod, V.K.; Pandit, A.B. Effect of various additives on enzymatic hydrolysis of castor oil. *Biochem. Eng. J.* **2009**, *47*, 93–99. [CrossRef]
19. Su, F.; Li, G.-L.; Fan, Y.-L.; Yan, Y.-J. Enhancing biodiesel production via a synergic effect between immobilized rhizopus oryzae lipase and novozym 435. *Fuel Process. Technol.* **2015**, *137*, 298–304. [CrossRef]
20. Aguieiras, E.C.; Cavalcanti-Oliveira, E.D.; Freire, D.M. Current status and new developments of biodiesel production using fungal lipases. *Fuel* **2015**, *159*, 52–67. [CrossRef]
21. Ondul, E.; Dizge, N.; Keskinler, B.; Albayrak, N. *Biocatalytic Production of Biodiesel from Vegetable Oils*; InTech: Rijeka, Croatia, 2015.
22. Tan, T.; Lu, J.; Nie, K.; Deng, L.; Wang, F. Biodiesel production with immobilized lipase: A review. *Biotechnol. Adv.* **2010**, *28*, 628–634. [CrossRef] [PubMed]
23. Simpliciano, C.; Clark, L.; Asi, B.; Chu, N.; Mercado, M.; Diaz, S.; Goedert, M.; Mobed-Miremadi, M. Cross-linked alginate film pore size determination using atomic force microscopy and validation using diffusivity determinations. *J. Surf. Eng. Mater. Adv. Technol.* **2013**, *3*, 1. [CrossRef]
24. Zhang, G.; Ma, J.; Wang, J.; Li, Y.; Zhang, G.; Zhang, F.; Fan, X. Lipase immobilized on graphene oxide as reusable biocatalyst. *Ind. Eng. Chem. Res.* **2014**, *53*, 19878–19883. [CrossRef]
25. Lu, J.; Deng, L.; Zhao, R.; Zhang, R.; Wang, F.; Tan, T. Pretreatment of immobilized candida sp. 99–125 lipase to improve its methanol tolerance for biodiesel production. *J. Mol. Catal. B Enzym.* **2010**, *62*, 15–18. [CrossRef]
26. Tan, T.; Nie, K.; Wang, F. Production of biodiesel by immobilized candida sp. Lipase at high water content. *Appl. Biochem. Biotechnol.* **2006**, *128*, 109–116. [CrossRef]
27. Liu, Y.; Yan, Y.; Hu, F.; Yao, A.; Wang, Z.; Wei, F. Transesterification for biodiesel production catalyzed by combined lipases: Optimization and kinetics. *AIChE J.* **2010**, *56*, 1659–1665. [CrossRef]
28. Huang, Y.; Zheng, H.; Yan, Y. Optimization of lipase-catalyzed transesterification of lard for biodiesel production using response surface methodology. *Appl. Biochem. Biotechnol.* **2010**, *160*, 504–515. [CrossRef] [PubMed]