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Surfactant Imprinting Hyperactivated Immobilized Lipase as Efficient Biocatalyst for Biodiesel Production from Waste Cooking Oil

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Abstract: Enzymatic production of biodiesel from waste cooking oil (WCO) could contribute to resolving the problems of energy demand and environment pollutions.In the present work, *Burkholderia cepacia* lipase (BCL) was activated by surfactant imprinting, and subsequently immobilized in magnetic cross-linked enzyme aggregates (mCLEAs) with hydroxyapatite coated magnetic nanoparticles (HAP-coated MNPs). The maximum hyperactivation of BCL mCLEAs was observed in the pretreatment of BCL with 0.1 mM Triton X-100. The optimized Triton-activated BCL mCLEAs was used as a highly active and robust biocatalyst for biodiesel production from WCO, exhibiting significant increase in biodiesel yield and tolerance to methanol. The results indicated that surfactant imprinting integrating mCLEAs could fix BCL in their active (open) form, experiencing a boost in activity and allowing biodiesel production performed in solvent without further addition of water. A maximal biodiesel yield of 98% was achieved under optimized conditions with molar ratio of methanol-to-WCO 7:1 in one-time addition in hexane at 40 °C. Therefore, the present study displays a versatile method for lipase immobilization and shows great practical latency in renewable biodiesel production.

Keywords: biodiesel; waste cooking oil; lipase immobilization; interfacial activation; functionalized magnetic nanoparticles

1. Introduction

Over the past decades, biodiesel has attracted great interest as a sustainable alternative for fossil fuels in virtue of the depletion of fossilized fuel resources and their environmental impacts [1]. Biodiesel is a renewable and clean energy, and possess favorable advantages in combustion emission like low emissions of CO, sulfur free, low hydrocarbon aroma, high cetanenumber, and high flash point [2].

The conventional chemical technologies for biodiesel production involve the use of acid or basic catalysts (i.e., NaOH, KOH, and H_2SO_4), thus numerous disadvantages are inescapable, for example, high corrosive procedure, high energy consumption, high quantities of waste pollution, and costly in efficient product separation processes [3]. Furthermore, in order to prevent the hydrolysis reaction and saponification, high quality oils are required, with low contents of water and free fatty acids [4].

Feedstocks used for biodiesel can be allocated five categories, including edible vegetable oils, non-edible plant oils, animal fats, microalgae oils, and waste oils [5]. The global application of first-generation biodiesel produced by using edible oils, was restricted due to food scarcity and high cost of the edible oils [6]. Biodiesel production from waste cooking oils (WCO) could be a promising and cost effective candidate in handling issues associated with energy crisis, environmental concerns, and total cost reduction of biodiesel production [7]. Moreover, 15 million tons of WCO are

produced annually throughout the world [8], bringing great challenge in reasonable management of such oils on account of environment concerns [9]. However, using WCO as raw material is quite challenging as it contains a high amount of free fatty acids (FFAs) and water which could hinder the homogeneous alkaline-catalyzed transesterification in conventional biodiesel production processes [10]. Complete conversion of these low-quality feedstocks like WCO could be accomplished in enzymatic biodiesel production without saponification. Therefore, enzyme-catalyzed transesterification has become a laudable potential alternative for biodiesel synthesis.

Particularly, lipases are foremost and efficient enzymes implemented in biodiesel production. Lipase-catalyzed process exhibits key advantages such as no soap formation, high-purity products, easy product removal, adaptable to different biodiesel feedstock, environmentally friendly, and mild operating conditions [5]. However, the commercialization of enzymatic biodiesel production remains complicated, because of high price and low stability of lipases as well as low reaction rate of biocatalysis. Heterogeneous enzyme-catalyzed transesterification using immobilized lipases is a possible solution to these problems [11].

Immobilization of enzymes has been investigated for many years, and lipase can generally be immobilized by various techniques such as cross-linking, adsorption, entrapment and encapsulation [12,13]. Thereinto, cross-linked enzyme aggregates (CLEAs) is a cheap and efficient strategy for enzyme immobilization, which has broad applicability over numerous enzyme classes. Owing to its outstanding resistance to organic solvents, extreme pH, and high temperatures, CLEAs has attracted growing attention in cost effective biocatalysis [14]. Nevertheless, small particle size and low mechanical stability of CLEAs could directly affect mass transfer and stability under operational conditions, thus accordingly cause problems in practical use [15]. An alternative approach for circumventing compressed construction of CLEAs is to use "smart" magnetic CLEAs (mCLEAs). Magnetic nanoparticles (MNPs) could provide enhanced stability over repeated uses, especially for enzymes having low amount of lysine residues on their surface. Besides, mCLEAs could perform easily separation using a permanent magnet, affording novel combinations of bioconversions and down-streaming processes, thus provide the necessary reduction in enzymecosts to enable commercial viability.

Among various types of nanomaterials, MNPs have attracted substantial attention in enzyme immobilizations. However, bare MNPs tend to aggregate due to their high surface energy and are easily oxidized in the air leading to loss of magnetism and dispersibility, thus limiting their exploitation in practical applications [16]. The surface modification with an organic or an inorganic shell is an appropriate strategy to address these issues. Due to their excellent biocompatibility, slow biodegradation, high surface area-to-volume-ratio, and unique mechanical stability, Hydroxyapatite (HAP) could be a proper inorganic surface coating for MNPs [17]. Moreover, HAP-coated MNPs can be easily functionalized with organosilanes, and consequently has great application potential in enzyme immobilization.

Burkholderia cepacia lipase (BCL)is one of the most widely used lipases in biocatalysis [18]. On account of its versatility to accommodate a wide variety of substrates, high heat resistance, and good tolerance to polar organic solvents, BCL has been extensively used in various biotechnological processes, especially for biodiesel production. The active site of BCL is shielded by a mobile element, called the lid or flap [19]. The displacement of lid or flap to closed or open position, which directly impacts the accessibility of active site, determines the enzyme in an in active or active conformation. In general, substrate access to the underlying active site is prohibited in its closed configuration. As the stabilization of the open conformation of all lipases could remarkably increase their catalytic activity, a favorable method to obtain highly active biocatalysts should try to immobilize lipases in their most active form (open conformation).

Generally, the preparation of immobilized enzyme with enhanced activity and stability is a persistent goal of the biotechnology industry to seek maximum profit. Therefore, developing a simple and efficient approach for lipase interfacial activation in immobilization is highly desirable.

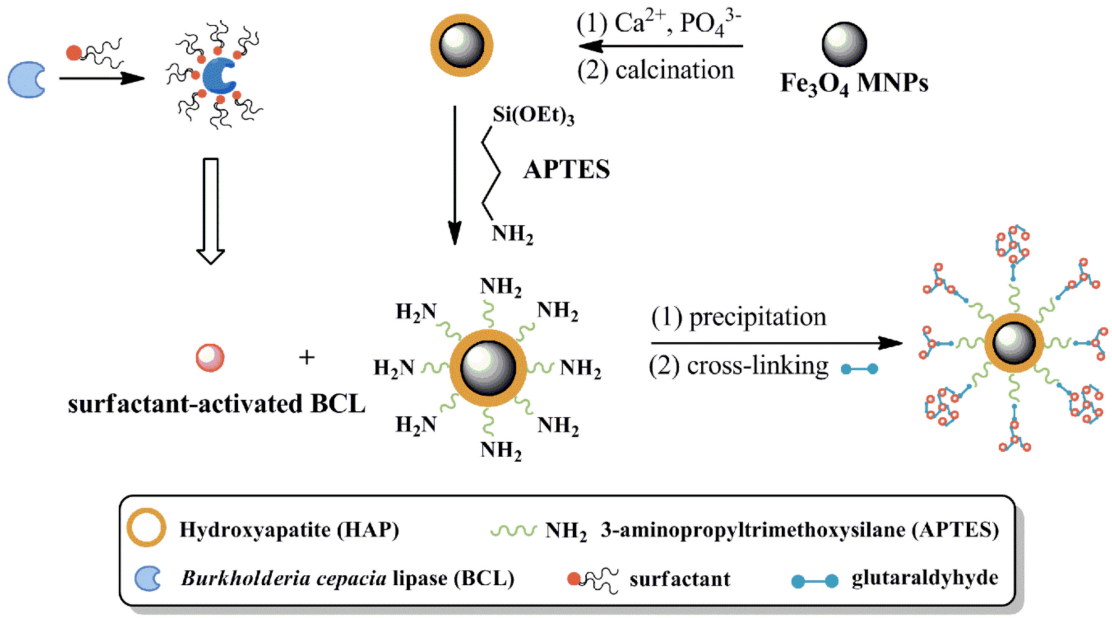
Bioimprinting is a commonly used method for achieving hyperactivation of lipases in organic media. The principle of bioimprinting is to "anchor" the enzyme in its active form, which could be achieved by binding with imprint molecules (such as surfactants, natural substrates, substrate analogs etc.). From an applied point of view, the dramatic hyperactivation of lipases by low concentrations of surfactants is an expeditious and facile method for lipase interfacial activation [20].

To develop an efficient and environmentally benign process for the biodiesel production from waste cooking oils, in the present study surfactant imprinting strategy on BCL was implemented in combination with mCLEAs immobilization using HAP-coated MNPs. Subsequent cross-linking could "lock" BCL in its favorable conformation, while HAP-coated MNPs could facilitate the recovery of immobilized BCL and simplify the biodiesel purification. To the best of our knowledge, this is the first report on BCL immobilization integrating surfactant imprinting and mCLEAs. The optimal conditions for mCLEAs preparation, along with the effect of different surfactants (anionic, cationic, and non-ionic) on the catalytic activity of BCL mCLEAs in transesterification were studied. The optimized surfactant-activated BCL mCLEAs was further used in transesterification of waste cooking oils to biodiesel. In addition, a detailed analysis of solvents, methanol-to-oil molar ratio, and temperatures on the yield of biodiesel production was presented. The results obtained in the research are expected to provide a reliable basis for further exploration of lipase immobilization and efficient biodiesel production in industry.

2. Results and Discussion

2.1. Preparation and Characterization of Immobilized Lipase

In this study, the prepared MNPs encapsulated by hydroxyapatite (HAP) were used as immobilization supports. The amino functionalization of HAP-coated MNPs was carried out using 3-aminopropyltrimethoxysilane (APTES) for efficient enzyme attachment. Typically, the preparation procedure of immobilization supports and surfactant-activated BCL mCLEAs were performed according to the scheme shown in Scheme 1. The prepared magnetic supports and immobilized BCL were characterized by fourier transform infrared spectroscopy (FT-IR), transmission electron microscope (SEM) and vibrating sample magnetometer (VSM).



Scheme 1. Preparation procedure of immobilization supports and surfactant-activated *Burkholderia cepacia* lipase (BCL) magnetic cross-linked enzyme aggregates (mCLEAs).

FTIR characterization was performed to investigate the chemical composition of functionalized MNPs and immobilized BCL. Spectra were recorded on over the region from 4000 to 400 cm^{-1}. As shown in Figure 1, the strong peak at 588 and 639 cm^{-1} corresponds to the stretching vibration of Fe-O bond. The characteristic absorption bands related to the HAP appease at 565 and 1044 cm^{-1}, which are assigned to phosphate groups [21]. In the IR spectrum of modified MNPs and BCL mCLEAs, the characteristic absorption bands related to the functional groups of HAP emerged clearly, which demonstrated the successful incorporation of MNPs with HAP. For all immobilized lipases, including BCL CLEAs, BCL mCLEAs and surfactant-activated BCL mCLEAs, the typical IR bands responsible for the lipase that were chemically covalent-bonded to the functionalized MNPs were observed at 1642 cm^{-1} for amide I (C=O stretching vibration) and at 1539 cm^{-1} for amide II (N-H bending vibration), respectively. Besides, compared with the results shown in Figure 1, aliphatic C-H stretch band at 2859 and 2927 cm^{-1}, corresponding to C-H stretching vibrations, are clearly observed in all immobilized lipases, which also indicated the successful loading of lipase.

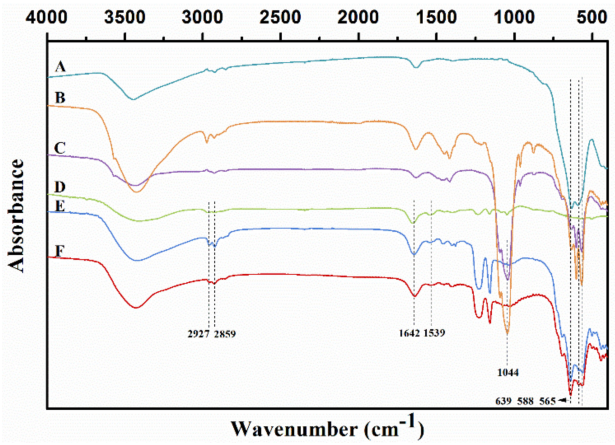


Figure 1. Spectra of (**A**) Fe_3O_4 MNPs, (**B**) hydroxyapatite coated magnetic nanoparticles (HAP-coated MNPs), (**C**) 3-aminopropyltrimethoxysilane (APTES)-HAP-coated MNPs, (**D**) BCL CLEAs, (**E**) BCL mCLEAs, (**F**) Triton-activated BCL mCLEAs.

In order to assess morphology, size and composition of functionalized MNPs and immobilized BCL, SEM images were collected and illustrated in Figure 2. As seen in Figure 2, bare Fe_3O_4 MNPs formed significantly dense agglomeration, because of their high surface energy and strong dipole-dipole interactions. It is obvious that the structure of Fe_3O_4 MNPs becomes looser and more evenly distributed after being functionalized with HAP (Figure 2B) and APTES (Figure 2C), suggesting that surface modification is favorable for preventing aggregation of Fe_3O_4 MNPs. At the same time, the rough surface of Fe_3O_4 MNPs also increased the surface area for attachment of enzyme.

The crucial structure factors in aggregated-based enzyme immobilization, including morphological topographies, structural arrangement and size, play an important role in affecting substrate affinity and stability of biocatalyst [22]. Besides, the particle size of enzymes is an important property of any heterogeneous catalysis since it can directly affect the diffusion of substrates and catalytic efficiency, especially in the internal enzymes of highly compact aggregates [23]. SEM images (Figure 2D) of standard BCL CLEAs revealed no defined morphologies and large size particles. Moreover, standard BCL CLEAs presented a uniform and compact surface with the presence of few tiny pores. On the contrary, after the incorporation of functionalized MNPs, BCL mCLEAs formed spherical structures and small particle sizes, which could reduce inner steric hindrance in closely packed CLEAs. It is noteworthy that the presence of functionalized MNPs displayed large active surface available for lipase immobilization, therefore were important for development of a stabilized enzyme-matrix. Furthermore, a loose and homodispersed structure of Triton-activated BCL mCLEAs was found in Figure 2F, suggesting that the formation of large aggregates were forbidden by the imprinting of

surfactants. From the SEM outcomes, it can be discerned that, thanks to the coating of surfactants, lipase could be uniformly dispersed on functionalized MNPs, which could contribute to a wider surface area with more catalytic sites and decrease the diffusion limit. Consequently, compared with standard BCL CLEAs, Triton-activated BCL mCLEAs could perform superior catalytic efficiency.

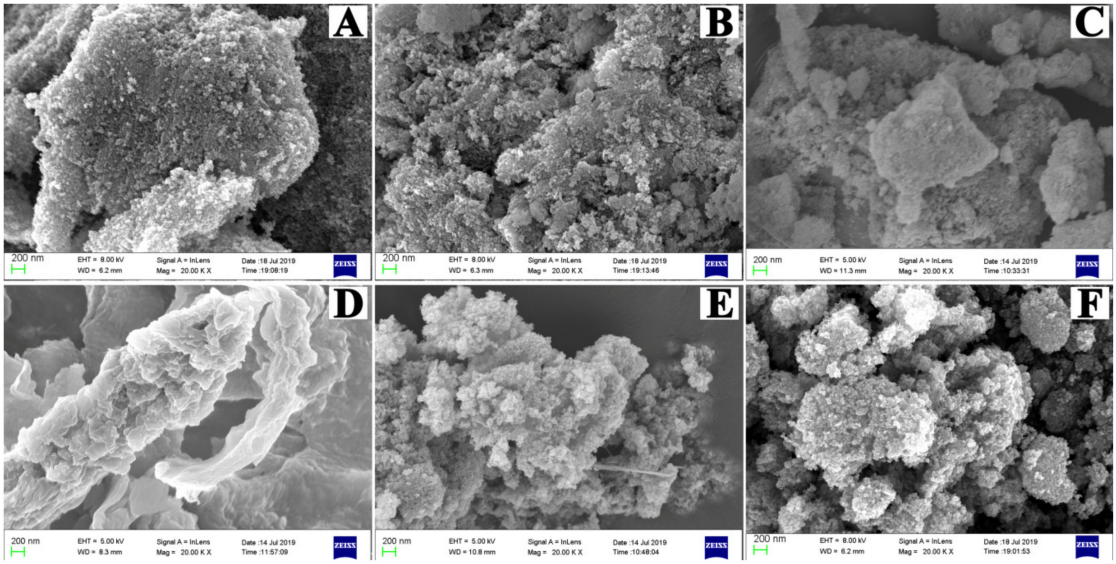


Figure 2. Images of(**A**) Fe_3O_4 MNPs, (**B**) HAP-coated MNPs, (**C**) APTES-HAP-coated MNPs, (**D**) BCL CLEAs, (**E**) BCL mCLEAs, (**F**) Triton-activated BCL mCLEAs.

The magnetic property of functionalized MNPs and immobilized BCL were measured using VSM. The hysteresis curves of the Fe_3O_4 MNPs, HAP-coated MNPs, APTES-HAP-coated MNPs, BCL mCLEAs and Triton-activated BCL mCLEAs shown in Figure 3, exhibited a perfect sigmoidal behavior, corresponding to a typical superparamagnetism.

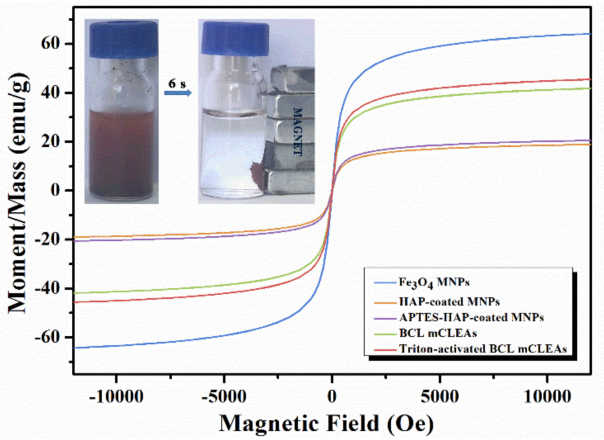


Figure 3. Hysteresis loops of Fe_3O_4 MNPs, HAP-coated MNPs, APTES-HAP-coated MNPs, BCL mCLEAs and Triton-activated BCL mCLEAs. The inner shows the easy magnetic separation of Triton-activated BCL mCLEAs in reaction mixture.

With further functionalization of MNPs, the saturation magnetization value decreased and correlated with the increase of the core-shell layer. Interestingly, it is obviously observed that the saturation magnetization value of Triton-activated BCL mCLEAs increased visibly compared to BCL mCLEAs. It might be due to the uniform dispersion of lipase on MNPs and availability of large surface area which decreased the shielding-effect of the out layer substances. As seen in Figure 3 (inner), Triton-activated BCL mCLEAs showed fast response (6s) to the external magnetic field andcould be

easily recovered from the reaction mixture. After removing the external magnetic field, the magnetic immobilized BCL redispersed rapidly by a slight shake, indicating good dispersion and efficient recyclability in industrial application.

2.2. Optimization of the Immobilization Conditions

In this study, the enzymes were precipitated by adding water-miscible organic solvents (acetone, ethanol and 2-propanol), PEG 800 and ammonium sulfate. The optimum precipitant was selected by measuring the transesterification activity of the corresponding BCL mCLEAs. Compared with free BCL, all BCL mCLEAs prepared using different precipitants performed higher transesterification activity in organic solvent. Among the protein precipitants evaluated, ammonium sulfate showed maximum recovery of activity (Figure 4a), therefore was further used in BCL immobilization.

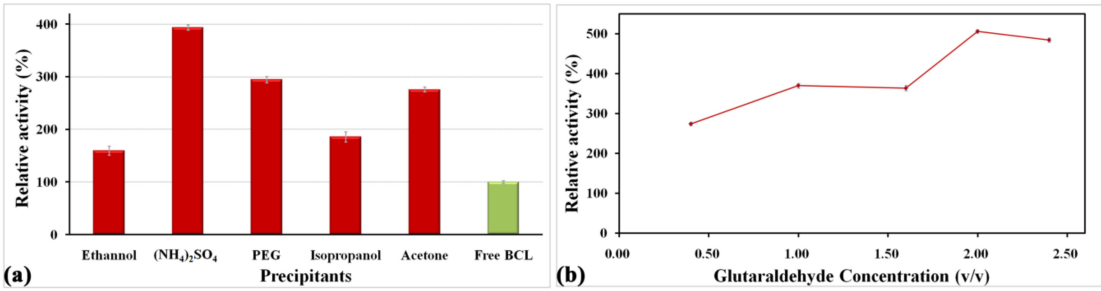


Figure 4. (**a**) Precipitant type and (**b**) glutaraldehyde concentration on the activities of BCL mCLEAs.

Traditionally, glutaraldehyde has been extensively used as the cross-linking agent to prepare CLEAs of various enzymes and exhibited a strong effect on activity and particle size of enzyme aggregates. The activity recovery of CLEAs greatly depends on the type of enzyme and the concentration of glutaraldehyde [24]. Lower glutaraldehyde concentration affects the cross-linking efficiency, which might result in enzyme leakage in immobilization, while excessive glutaraldehyde can induce the flexibility of enzymes and the active site availability, consequently, decreasing the activity recovery of CLEAs [25,26]. In this study, the influence of glutaraldehyde concentration on activity of BCL mCLEAs was investigated by using various concentrations of glutaraldehyde in cross-linking. As shown in Figure 4b, the optimum glutaraldehyde concentration of BCL mCLEAs was 2.0% (v/v).

2.3. Hyperactivation of BCL mCLEAs with Surfactants

A pivotal challenge in lipase immobilization is to open the lid of lipases and fix their open form for the exposure of active site. Surfactant imprinting is an efficient approach to activate lipases by facilitating lid-opening. Like other lipases, BCL also consists of a mobile element at the surface, which composed of two helical elements (a5- and a9-helices) and covers the active site [18]. To improve the catalytic performance of BCL mCLEAs, BCL was imprinted in the presence of surfactants prior to immobilization. Thus, four different surfactants with different properties (cationic, anionic and non-ionic) were investigated for modulating the activity of BCL mCLEAs in biodiesel production. As seen in Figure 5, Triton X-100 exhibited maximum effect on the enhancement of lipase activity in low surfactant concentration, while the addition of sodium bis-2-(ethylhexyl) sulfosuccinate (AOT) showed the least influence. The optimal surfactant in proper concentration acting as a bipolar agent, could simulate the amphiphilic environment to benefit the exposure of hydrophobic regions in the active site. Meanwhile, surfactants may also promote the dissociation of large aggregates formed, thus slightly increase the enzymatic activity of lipase (Figure 2).

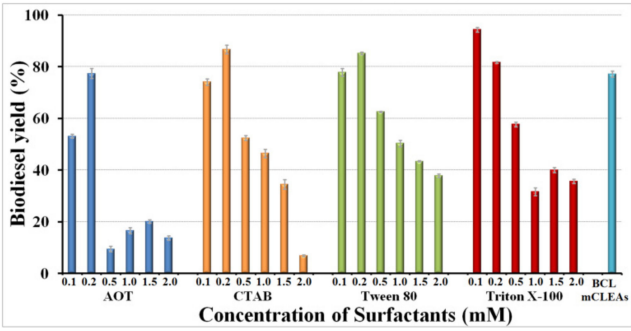


Figure 5. Four different surfactants activation on activity of surfactant-activated BCL mCLEAs in biodiesel production.

However, the increase of surfactant concentration led to gradual decrease of biodiesel yield in all cases, indicating that surfactants showed positive and negative effect on the activity of lipase. Additional detergent molecules may bind to the active site region of lipase, blocking the substrate access, inducing inhibition [27]. Compared with ionic surfactants (AOT and cetrimonium bromide (CTAB)), nonionic surfactants (Triton X-100 and Tween 80) were preferred aiming at regulating the activity of BCL (Figure 5). As the main interaction between the enzyme and nonionic surfactants is hydrophobic interaction while anionic or cationic surfactants perform electrostatic interactions [28], mild hydrophobic interaction between BCL and the surfactant might be important to trigger the interfacial activation mechanism. Therefore, nonionic Triton X-100 and Tween 80 were further studied to confirm the optimal amphiphile and surfactant concentration. As performed in Figure 6, the maximum hyperactivation of BCL mCLEAs was observed in the pretreatment of BCL with 0.1 mM Triton X-100, and the optimal Triton-activated BCL mCLEAs were used for further experiments.

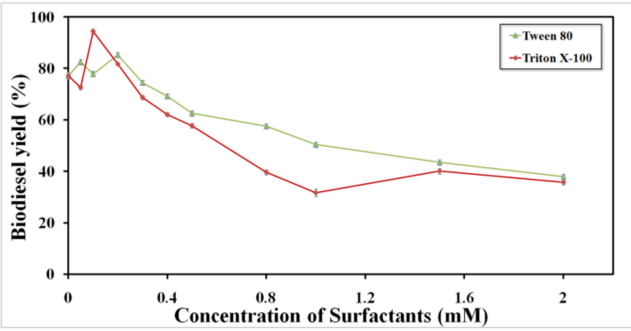


Figure 6. Surfactants (Triton X-100 and Tween 80) concentration in surfactant-activated BCL mCLEAs preparation.

2.4. Biodiesel Production

For the economic feasibility of biodiesel production, solvents, methanol-to-oil molar ratio, and reaction temperature are important variables to optimize for transesterification step. As a result of oxidative reactions occurring during cooking and long-term storage in air, WCO generally exhibits a dramatic increase in viscosity and saponification value [7]. Compared to the fresh oil, high viscose WCO is not favored in biodiesel production. Using solvents could reduce the viscosity of the reaction medium and decrease the diffusion limitations, while it might also directly affect the enzyme structure and activity. In general, hydrophobic solvents could promote the interface and stabilize lipases on their open assembly, causing the hyperactivation of these enzymes. To select the most suitable medium, five hydrophobic solvents commonly used in transesterification were tested in biodiesel production (Figure 7a). It can be clearly seen that biodiesel yield is remarkably dependent on the type of solvent. Overall, Triton-activated BCL mCLEAs exhibited higher activity than BCL mCLEAs and free BCL in all the solvents tested, and the changing trend of their activity in various solvents was accord with

BCL mCLEAs and free BCL. In case of Triton-activated BCL mCLEAs, the best results were achieved using n-hexane with a yield of up to 94% biodiesel, which was 3.3-fold higher than that in free BCL catalyzed reaction. Interestingly, surfactant hyperactivation in combination with immobilization could fasten lipase in their active conformation, allowing biodiesel production performed in solvent without further addition of water, which was in accordance with earlier reports [29,30].

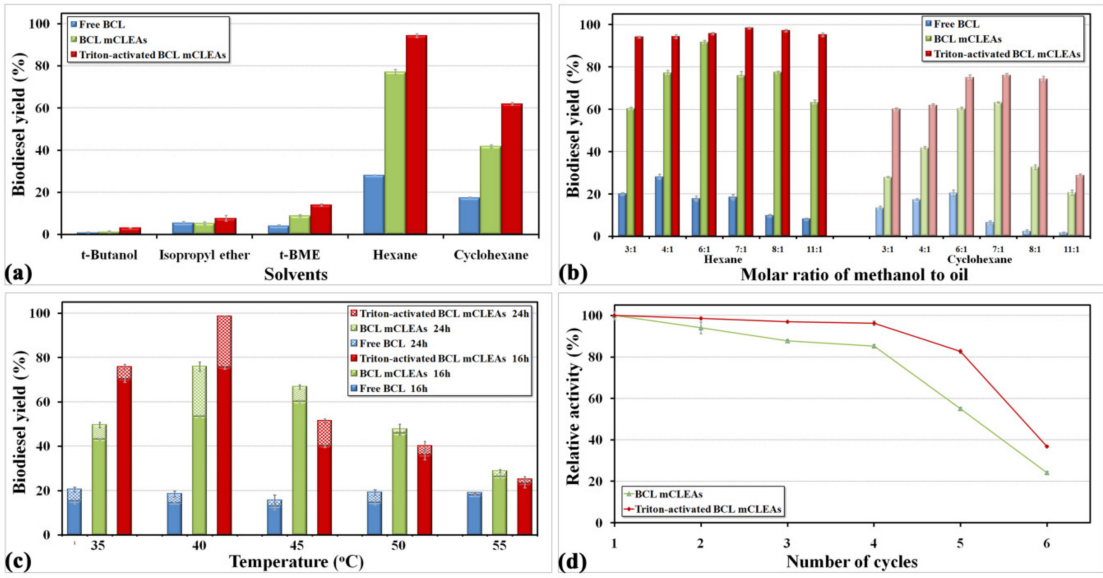


Figure 7. Reaction parameters on biodiesel production catalyzed by free BCL, BCL mCLEAs and Trion-activated BCL mCLEAs and reusability of immobilized BCL. (**a**) Solvents, (**b**) molar ratio of methanol to oil, (**c**) temperature, (**d**) reusability.

The methanol:oil molar ratio can have a significant effect on the reaction yield because excess methanol increases the reaction rate and drives high yield of biodiesel, while a high concentration of methanol leads to inactivation of lipases. In this study, experiments were performed at different molar ratios of methanol to WCO ranging from 3:1 (stoichiometric ratio) to 11:1 both in hexane and cyclohexane with methanol added only once. As shown in Figure 7b, Triton-activated BCL mCLEAs exhibited higher biodiesel yields in one-time addition of methanol under all the experimental conditions, especially when hexane was used as solvent. Meanwhile, owing to the significant inhibitory effect of excess methanol in one time addition, free BCL showed low activity in biodiesel production. It is worth noting that yields of Triton-activated BCL mCLEAs catalyzed reactions in hexane exceeded 90% in a wide range of methanol-to-WCO ratio, while the reaction yield of BCL mCLEAs catalyzed reaction decreased with methanol-to-WCO ratio exceeding 6:1. Consequently, it can be confirmed that surfactants pretreatment provided not only hyperactivation but also protection to lipases from denaturation in excess methanol. In addition, the maximum biodiesel yield was observed at a methanol-to-WCO ratio of 7:1 for Triton-activated BCL mCLEAs. Consequently, the minimal stoichiometric methanol-to-WCO ratio of 7:1 was chosen in further experiments.

Most of the enzymatic transesterification depends on temperature, which could enhance reaction rate and improve the dispersion of immobilized particles in reaction medium with better mass transfer between the reactants [31]. However, thermal denaturation of the enzyme might occur with elevation of temperature, typically according to the property of enzyme and immobilized methods. The effect of temperature on the yield of biodiesel during the transesterification of WCO has been investigated over a temperature range from 35 to 55 °C. According to Figure 7c, the optimum operational temperature for Triton-activated BCL mCLEAs and BCL mCLEAs was 40 °C, with biodiesel yields of 98% and 76% respectively after 24 h, and further increase of temperature will result in decrease of biodiesel yields simultaneously. Besides, Triton-activated BCL mCLEAs showed better activity below 40 °C, while BCL mCLEAs performed higher biodiesel yield over 40 °C. The suitable covalent cross-linking

with functionalized MNPs provided extra structure stabilization in mCLEAs, requiring much more energy to the disruption of this stable structure than free enzyme [32]. Nevertheless, the accessible active site of lipases achieved by surfactants pretreatment might be more sensitive to high temperature denaturation [33].

In summary, the optimal reaction conditions for Triton-activated BCL mCLEAs catalyzed transesterification of WCO are as follows: hexane used as solvent, molar ratio of methanol-to-WCO 7:1 in one-time addition, reaction temperature 40 °C. To verify the feasibility of the whole process at a larger scale, transesterification of WCO were performed under optimal conditions adding proper amount of Triton-activated BCL mCLEAs (the initial content of BCL was 240 mg in immobilization) to a mixture of 1 g WCO in 20 mL hexane. The biodiesel yield reached 94% after shaking at 40 °C for 48 h. Triton-activated BCL mCLEAs showed good activity and stability under higher oil content, indicating the possibility of its scale-up application in bioreactor systems.

2.5. Reusability

Reusability of immobilized enzyme is a chief criterion for its cost-effective use for potential industry applications. The utilization of functionalized MNPs facilitates the consequent reuse of immobilized enzyme. To investigate the reusability of BCL mCLEAs and Triton-activated BCL mCLEAs, the immobilized lipases were recovered by magnetic separation, and applied in the consecutive batches of biodiesel reactions under optimized conditions. Assessments of the operational stability were analyzed for 6 cycles and presented in Figure 7d. As observed, Triton-activated BCL mCLEAs showed no significant loss in the catalytic activity after subsequent consecutive reuse for 4 cycles, and kept 82% relative activity after continuous running 5 cycles. Meanwhile, the relative activity of BCL mCLEAs was 55% after 5 cycles, implying that BCL could possess good long-term stability with surfactant pretreatment. The protein denaturation in one time addition of methanol and byproduct inhibition might be account for the decrease in biodiesel yield in long-term reuses [34].

3. Materials and Methods

3.1. Materials

Burkholderia cepacia lipase (powder, Amano Lipase PS, ≥3000 U/g) and fatty acid methyl ester standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). Also, 3-aminopropyl triethoxysilane (APTES), glutaraldehyde (25%, v/v) and 2-phenyl ethanol (>98%, CP) were supplied by Aladdin (Shanghai, China). Sodium bis-2-(ethylhexyl) sulfosuccinate (AOT) were procured from Acros (USA). Waste cooking oil (WCO) was obtained from local restaurant around Ningxia University campus (Yinchuan, China) with the following fatty acid compositions: 10.48% palmitic acid, 15.04% stearicacid, 38.44% oleic acid, 23.76% linoleic acid, and 1.72% linolenic acid. The WCO sample was filtered to separate impurities and solids in the oil. The physical properties of WCO are saponification value of 197.3 mg KOH/g, acid value of 4.37 mg KOH/g, and average molecular weight of 870.9 g/mol. All other chemicals were of analytical or chromatographical grade and used as purchased.

3.2. Preparation of Magnetic Support

Preparation of HAP-coated MNPs was carried out according to the previously reported method [35]. Initially, MNPs cores were prepared by the conventional co-precipitation method. Typically, $FeCl_2{\cdot}4H_2O$ (1.1 g) and (3.0 g) of $FeCl_3{\cdot}6H_2O$ were dissolved in 90 mL deionized water under the protection of argon, with subsequent addition of 25% ammonia solution (30 mL) under vigorous stirring at room temperature. After stirring for 30 min, a 60 mL aqueous solution composed of $Ca(NO_3)_2{\cdot}4H_2O$ (7.1 g) and $(NH_4)_2HPO_4$ (2.3 g) adjusted to pH=11 was added drop wise to the above suspension under continuous stirring. Subsequently, the resultant mixture was heated to 90 °C and stirred for 2 h. After cooling to room temperature and aging in the mother solution overnight, the obtained

precipitates were washed several times with deionized water until neutral and lyophilized for 12 h. The HAP-coated MNPs were obtained by calcining the materials in air at 300 °C for 3 h.

To obtain 3-aminopropyl trimetoxysilane functionalized HAP-coated MNPs (APTES-HAP-coated MNPs), HAP-coated MNPs (1.0 g) were suspended in a solution composed of 30 mL anhydrous toluene and 0.44 g of APTES. The mixture was refluxed under Ar atmosphere for 12 h, and then washed several times with ethanol, magnetically separated, and subsequently lyophilized prior to use.

3.3. Lipase Immobilization

BCL mCLEAs were produced according to the procedure described in Scheme 1. Firstly, 10 mg of APTES-HAP-MNPs were dispersed in 1 mL of BCL solution (10 mg/mL, 0.1 M phosphate buffer, pH 7.0) and shaken for 15 min at 30 °C. Then 5 mL of precipitant was added with stirring at 4 °C for 30 min. After precipitation, glutaraldyhyde was added drop wise into the suspension and stirred for 3 h at 30 °C. Afterwards, BCL mCLEAs were collected by centrifugation and washed thrice with phosphate buffer and deionized water, lyophilized and finally stored at 4 °C.

During optimization of the immobilization conditions, the effects of precipitants (acetone, ethanol, isopropanol, PEG 800 (1 g/mL), and saturated ammonium sulfate solution) and concentration of glutaraldehyde on the activity recovery of BCL mCLEAs were investigated.

The surfactant-activated BCL mCLEAs was prepared using cationic (CTAB), anionic (AOT) and nonionic (Tween 80 and Triton Triton X-100) surfactants at various concentrations. Then, 1 mL of BCL solution and appropriate amount of surfactant were mixed and stirred at 4 °C for 30 min. After incubated for 24 h at 4 °C, the suspended solution was sequentially used for BCL mCLEAs preparation under optimal conditions.

3.4. Characterization

The prepared support matrix and immobilized lipase described above were characterized using FTIR, SEM and VSM. The Fourier transform infrared (FTIR) spectra were acquired using a Perkin Elmer Frontier spectrometer (Spectrum Two, Waltham, MA, USA) equipped with an Attenuate Total Reflection (ATR) accessory. Samples were analyzed as KBr pellets in the range of 400 to 4000 cm^{-1} at a resolution of 0.5 cm^{-1}. The morphology of the particle surface was observed using a scanning electron microscope (SEM, Sigma HD, ZEISS, Germany), with deposition of a thin coating of gold onto the samples prior to analyses. The magnetic properties were detected by a vibrating sample magnetometer (VSM, MicroSense EZ9, Lowell, MA, USA) at room temperature.

3.5. Activity Assay

In studying the optimal conditions for BCL mCLEAs preparation, the enzymatic transesterification activities of free lipase and immobilized BCLs were assayed via transesterification reaction of 2-phenyl ethanol with vinyl acetate according to the method introduced previously [36]. The reaction mixture contained 10 mg of 2-phenylethanol, 1 mL of vinyl acetate and 10 mg of lipase (the initial content of BCL was 10 mg in preparing BCL CLEAs and BCL mCLEAs), and the reactions were carried out at 30 °C with continuous shaking at 220 rpm. After 24 h of reaction, samples were withdrawn and analyzed by high-performance liquid chromatography (HPLC). All experiments were repeated at least three times. The relative activity of BCL mCLEAs was calculated with the following equation:

$$\text{Relative activity } (\%) = \frac{\text{Transesterification yield of immobilized BCL}}{\text{Trasesterification yield of free BCL}} \times 100$$

3.6. Enzymatic Transesterification for Biodiesel Production

The transesterification of WCO were carried out at 40 °C in a 10 mL screw-capped vessel for 24 h with continuous shaking at 220 rpm. Unless otherwise stated, a typical reaction mixture consisted of 50 mg WCO, 2.0 mL hexane, 10 mg of lipase (the initial content of BCL was 10 mg in preparing

BCL CLEAs and BCL mCLEAs) and methanol using methanol: oil molar ratio of 4:1. Single factor optimization was conducted to determine optimal reaction parameters for transesterification of WCO to biodiesel. Various conditions including kinds and concentration of surfactants, solvents, molar ratio of methanol to oil and temperature (°C) were investigated. The transesterification reaction of large scale with 1 g WCO were carried out as described in Section 2.4. All biodiesel reactions were performed in dried solvents without any water added. The yield of biodiesel (20 μL) was analyzed in different time intervals using gas chromatography.

3.7. Analytical Methods

HPLC was conducted with Shimadzu LC-2010A HT apparatus using C18 column (UltimateXB-C18, 5 μm, 4.6 × 150 mm, Welch). The samples were analyzed with a mixture of MeOH/water = 80:20 (v/v) as eluent at 0.8 mL/min for 9 min at 254 nm.

Fatty acid methyl esters (FAMEs) were analyzed by a Fuli9790 plus gas chromatography (Fuli, Zhejiang, China) fitted with a flame ionization detectorcity (FID, Zhejiang, China), and a KB-FFAP column (30 m × 0.32 mm × 0.25 μm). Nitrogen gas was a carrier at continues flow of 1.0 mL/min. The oven (Zhejiang, China) temperature was set and at 160 °C maintained for 2 min, then a heating ramp was applied up to 240 °C at a rate of 10 °C /min, and the temperature of the oven was maintained at 240 °C for 15 min. The temperatures of the injector (Zhejiang, China) and the detector (Zhejiang, China) were set at 270 and 280 °C, respectively. Methyl tridecanoate was used as internal standard, and the biodiesel yield (%) was calculated by peaks area of standard FAME peaks.

3.8. Reusability

The reusability of Triton-activated BCL mCLEAs and BCL mCLEAs for the transesterification of WCO were also investigated under optimal conditions. After each batch reaction, immobilized BCL was recovered by magnetic separation and washed with n-hexane. The washed biocatalyst was reused consecutively in repetitive cycles. The biodiesel yield of the first reaction was set as 100% and the FAMEs yield in the subsequent reactions was calculated accordingly.

4. Conclusions

A facile and effectual surfactant imprinting method to expose the lipase active site integrating amino functionalized HAP-coated MNPs was established to immobilize CLEAs of BCL attaining enhanced activity and stability. The as-prepared Triton-activated BCL mCLEAs was subsequent processed in enzymatic transesterification of waste cooking oil for biodiesel production, and showed 98% biodiesel yield under optimal conditions, which was 5.3-fold higher than the free lipase. This study proved that hyperactivation with surfactant could significantly improve the resistance of lipase to methanol in one-time addition, when compared to BCL mCLEAs and free BCL. In addition, surfactant imprinting in combination with immobilization could fasten lipase in their active conformation, allowing biodiesel production performed in solvent without further addition of water, and thus displayed priority in downstream purification of biodiesel over ordinary immobilization methods. Besides, the green immobilization with functionalized MNPs facilitates fast and easy recovery of lipase, and the corresponding immobilized BCL was reused for 4 cycles without significant loss in the catalytic activity. Furthermore, this work provides a promising approach for immobilization of other lipases, which can be used with success in green and clean production processes.

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References

1. Mahmudul, H.M.; Hagos, F.Y.; Mamat, R.; AbdulAdam, A.; Ishak, W.F.W.; Alenezi, R. Production, characterization and performance of biodiesel as an alternative fuel in diesel engines-a review. *Renew. Sustain. Energy Rev.* **2017**, *72*, 497–509. [CrossRef]
2. Laesecke, J.; Ellis, N.; Kirchen, P. Production, analysis and combustion characterizationof biomass fast pyrolysis oil-biodiesel blends for use in diesel engines. *Fuel* **2017**, *199*, 346–357. [CrossRef]
3. Jamil, F.; Alhaj, L.; Almuhtaseb, A.H.; Baawain, M.; Rashid, U.; Ahmad, M.N.M. Current scenario of catalysts for biodieselproduction: A critical review. *Rev. Chem. Eng.* **2018**, *34*, 267–297. [CrossRef]
4. Banković-Ilić, I.B.; Stamenković, O.S.; Veljković, V.B. Biodiesel production fromnon-edible plant oils. *Renew. Sustain. Energy Rev.* **2012**, *16*, 3621–3647. [CrossRef]
5. Hama, S.; Noda, H.; Kondo, A. How lipase technology contributes to evolution ofbiodiesel production using multiple feedstocks. *Curr. Opin. Biotechnol.* **2018**, *50*, 57–64. [CrossRef]
6. Gebremariam, S.N.; Marchetti, J.M. Economics of biodiesel production: Review. *Energy Convers. Manag.* **2018**, *168*, 74–84. [CrossRef]
7. Moazeni, F.; Chen, Y.-C.; Zhang, G. Enzymatic transesterification for biodiesel production from usedcooking oil, a review. *J. Clean. Prod.* **2019**, *216*, 117–128. [CrossRef]
8. Lopresto, C.; Naccarato, S.; Albo, L.; De Paola, M.; Chakraborty, S.; Curcio, S.; Calabro, V. Enzymatic transesterification of waste vegetable oil to producebiodiesel. *Ecotoxicol. Environ. Saf.* **2015**, *121*, 229–235. [CrossRef]
9. Chhetri, A.; Watts, K.; Islam, M. Waste cooking oil as an alternate feedstock forbiodiesel production. *Energies* **2008**, *1*, 3–18. [CrossRef]
10. Sabudak, T.; Yildiz, M. Biodiesel production from waste frying oils and itsquality control. *Waste Manag.* **2010**, *30*, 799–803. [CrossRef]
11. Kim, K.H.; Lee, O.K.; Lee, E.Y. Nano-immobilized biocatalysts for biodiesel production from renewable and sustainable resources. *Catalysts* **2018**, *8*, 68. [CrossRef]
12. Facin, B.R.; Melchiors, M.S.; Valério, A.; Oliveira, J.V.; Oliveira, D. Driving immobilized lipases as biocatalysts: 10 years state of the art and future prospects. *Ind. Eng. Chem. Res.* **2019**, *58*, 5358–5378. [CrossRef]
13. Filho, D.G.; Silva, A.G.; Guidini, C.Z. Lipases: Sources, immobilization methods, and industrial applications. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 7399–7423. [CrossRef]
14. Xu, M.-Q.; Wang, S.-S.; Li, L.-N.; Gao, J.; Zhang, Y.-W. Combined cross-linked enzyme aggregatesas biocatalysts. *Catalysts* **2018**, *8*, 460. [CrossRef]
15. Sheldon, R.A. CLEAs, combi-CLEAs and 'smart' magnetic CLEAs: Biocatalysis in a bio-based economy. *Catalysts* **2019**, *9*, 261. [CrossRef]
16. Liu, D.-M.; Chen, J.; Shi, Y.-P. Advances on methods and easy separated support materials forenzymes immobilization. *TrAC-Trends Anal. Chem.* **2018**, *102*, 332–342. [CrossRef]
17. Izadia, A.; Meshkinia, A.; Entezari, M.H. Mesoporous superparamagnetic hydroxyapatite nanocomposite: Amultifunctional platform for synergistic targeted chemo-magnetotherapy. *Mater. Sci. Eng. C Mater.* **2019**, *101*, 27–41. [CrossRef]
18. Sánchez, D.A.; Tonetto, G.M.; Ferreira, M.L. Burkholderia cepacia lipase: A versatile catalyst in synthesisreactions. *Biotechnol. Bioeng.* **2018**, *115*, 6–24. [CrossRef]
19. Barbe, S.; Lafaquiere, V.; Guieysse, D.; Monsan, P.; Remaud-Siméon, M.; Andre, I. Insights into lid movements of Burkholderia cepacialipase inferred from molecular dynamics simulations. *Proteins* **2009**, *77*, 509–523. [CrossRef]
20. Gabriele, F.; Spreti, N.; Giacco, T.D.; Germani, R.; Tiecco, M. Effect of surfactant structure on the superactivity of Candida rugosalipase. *Langmuir* **2018**, *34*, 11510–11517. [CrossRef]
21. Zhang, N.; Gao, T.; Wang, Y.; Wang, Z.; Zhang, P.; Liu, J. Environmental pH-controlledloading and release of protein on mesoporous hydroxyapatite nanoparticles forbone tissue engineering. *Mater. Sci. Eng. C Mater.* **2015**, *46*, 158–165. [CrossRef]

22. Kannan, S.; Marudhamuthu, M. Development of chitin cross-linked enzyme aggregates of L-methioninase for upgraded activity, permanence andapplication as efficient therapeutic formulations. *Int. J. Biol. Macromol.* **2019**, *141*, 218–231. [CrossRef]
23. Shaarani, S.M.; Jahim, J.M.; Rahman, R.A.; Idris, A.; Murad, A.M.A.; Illias, R.M. Silanized maghemite for cross-linked enzyme aggregates of recombinantxylanase from Trichoderma reesei. *J. Mol. Catal. B Enzym.* **2016**, *133*, 65–76. [CrossRef]
24. Rehman, S.; Bhatti, H.N.; Bilal, M.; Asgher, M. Cross-linked enzyme aggregates (CLEAs) of Pencilluim notatum lipaseenzymewith improved activity, stability and reusabilitycharacteristics. *Int. J. Biol. Macromol.* **2016**, *91*, 1161–1169. [CrossRef]
25. Cui, J.; Lin, T.; Feng, Y.; Tan, Z.; Jia, S. Preparation of spherical cross-linked lipaseaggregates with improved activity, stabilityand reusability characteristic in water-in-ionicliquid microemulsion. *J. Chem. Technol. Biotechnol.* **2017**, *92*, 1785–1793. [CrossRef]
26. Yamaguchi, H.; Kiyota, Y.; Miyazaki, M. Techniques for preparation of cross-linked enzyme aggregates and their applications in bioconversions. *Catalysts* **2018**, *8*, 174. [CrossRef]
27. Bañó, M.C.; González-Navarro, H.; Abad, C. Long-chain fatty acyl-CoA esters induce lipase activation in the absence of a water-lipid interface. *BBA Mol. Cell Biol. Lipids* **2003**, *1632*, 55–61. [CrossRef]
28. Delorme, V.; Dhouib, R.; Canaan, S.; Fotiadu, F.; Carriere, F.; Cavalier, J.F. Effects of surfactants on lipase structure, activity, and inhibition. *Pharm. Res.* **2011**, *28*, 1831–1842. [CrossRef]
29. Mukherjee, J.; Gupta, M.N. Molecular bioimprinting of lipases with surfactantsand its functional consequences in low water media. *Int. J. Biol. Macromol.* **2015**, *81*, 544–551. [CrossRef]
30. Zhang, W.-W.; Yang, X.-L.; Jia, J.Q.; Wang, N.; Hu, C.-L.; Yu, X.-Q. Surfactant-activated magnetic cross-linked enzyme aggregates (magnetic CLEAs) of Thermomyces lanugunosus lipase for biodiesel production. *J. Mol. Catal. B Enzym.* **2015**, *115*, 83–89. [CrossRef]
31. Lee, H.V.; Yunus, R.; Juan, J.C.; Taufiq-Yap, Y.H. Process optimization design forjatropha-based biodiesel production using response surface methodology. *Fuel Process. Technol.* **2011**, *92*, 2420–2428. [CrossRef]
32. Dong, T.; Zhao, L.; Huang, Y.; Tan, X. Preparation of cross-linked aggregates ofaminoacylase from Aspergillus melleus by using bovine serum albumin as aninert additive. *Bioresour. Technol.* **2010**, *101*, 6569–6571. [CrossRef]
33. Rehm, S.; Trodler, P.; Pleiss, J. Solvent-induced lid opening in lipases: A molecular dynamics study. *Protein Sci.* **2010**, *19*, 2122–2130. [CrossRef]
34. Xie, W.; Huang, M. Immobilization of Candida rugosa lipase onto graphene oxide Fe3O4nanocomposite: Characterization and application for biodiesel production. *Energy Convers. Manag.* **2018**, *159*, 42–53. [CrossRef]
35. Xie, W.; Zang, X. Covalent immobilization of lipase onto aminopropyl-functionalizedhydroxyapatite-encapsulated-γ-Fe_2O_3 nanoparticles: A magneticbiocatalyst for interesterification of soybean oil. *Food Chem.* **2017**, *227*, 397–403. [CrossRef]
36. Zhang, W.-W.; Yang, H.-X.; Liu, W.-Y.; Wang, N.; Yu, X.-Q. Improved performance of magnetic cross-linked lipase aggregates by interfacial activation: A robustand magnetically recyclable biocatalyst for transesterification of Jatropha oil. *Molecules* **2017**, *22*, 2157. [CrossRef]