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Review

Characterization of the lipase immobilized on Mg–Al hydrotalcite for biodiesel

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ABSTRACT

Immobilization of *Saccharomyces cerevisiae* lipase by physical adsorption on Mg–Al hydrotalcite with a Mg/Al molar ratio of 4.0 led to a markedly improved performance of the enzyme. The immobilized lipase retained activity over wider ranges of temperature and pH than those of the free lipase. The immobilized lipase retained more than 95% relative activity at 50 °C, while the free lipase retained about 88%. The kinetic constants of the immobilized and free lipases were also determined. The apparent activation energies (E_a) of the free and immobilized lipases were estimated to be 6.96 and 2.42 kJ mol^{-1}, while the apparent inactivation energies (E_d) of free and immobilized lipases were 6.51 and 6.27 kJ mol^{-1}, respectively. So the stability of the immobilized lipase was higher than that of free lipase. The water content of the oil must be kept below 2.0 wt% and free fatty acid content of the oil must be kept below 3.5 mg KOH g [oil]$^{-1}$ in order to get the best conversion. This immobilization method was found to be satisfactory to produce a stable and functioning biocatalyst which could maintain high reactivity for repeating 10 batches with ester conversion above 81.3%.

Contents

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1. Introduction

Biodiesel fuel, which consists of the simple alkyl esters of fatty acids (preferentially methyl esters), has got a growing interest as an alternative to diesel fuels made from renewable sources. Transesterification of vegetable oils for the production of fatty acid alkyl esters is a well-established industrial process. The conventional biodiesel technology involves the use of an inorganic base or acid catalyst at near the boiling temperatures of the triglyceride/alcohol mixture. The removal of catalyst is through neutralization and eventual separation of salt from the product esters, which is difficult to achieve [1,2]. To minimize homogeneous process problems, attempts to use heterogeneous catalyst systems in alcoholysis of triglycerides have been made. These catalysts greatly simplify the post-treatment of the products (separation and purification). They can be easily separated from the system at the end of the reaction and may also be reused. Besides, the use of heterogeneous catalysts does not produce soaps through free fatty acid neutralization or triglyceride saponification. A large number of heterogeneous catalysts have been reported in literature, including enzymes, zeolites, clays, ion-exchange resins, hydrotalcites and oxides [3,4].

Although the enzymatic process is still not commercially developed, enzymatic conversion of triglycerides has been suggested as a realistic alternative to the conventional physiochemical methods [2,3]. Enzymatic transesterification of triglycerides offers an environmentally more attractive option to the conventional process. However, the high cost of the enzymes often makes the enzymatic processes economically unattractive [2]. Enzyme immobilization technology may be an effective means for enzyme reuse and to improve its activity and stability [5]. The supports used for immobilizing enzyme should possess mechanical strength, microbial resistance, thermostability, chemical durability, chemical functionality, low cost, hydrophylicity, regenerability and a high capacity of enzyme. The various immobilization protocols used with enzymes have been extensively studied. Techniques for immobilization have been broadly classified into four categories, namely adsorption, covalent binding, entrapment and microencapsulation [6]. Immobilization of enzyme through physical method is still the most commonly used because it is the easiest to perform and the least expensive to prepare solid-support biocatalysts. In this method, the forces between a support and the enzymes include hydrogen bonding, Van der Waals forces and hydrophobic interactions [6,7]. Lipases are able to effectively catalyze the transesterification of triglycerides in either aqueous or non-aqueous systems and have been successfully immobilized on many different types of carriers, such as hydrotalcite, celite, kaolin, metal oxide, silica gel, and chitosan [5–9]. In our laboratory, an extracellular lipase produced by *Saccharomyces cerevisiae* was shown to be a potentially useful biocatalyst for the transesterification. Thus, it was considered a promising enzyme for future application in producing biodiesel.

In this article, several kinds of immobilization materials were employed as supports to immobilize lipase from *S. cerevisiae* via physical adsorption. Degrees of immobilization of diverse materials and catalytic properties of immobilized lipases were investigated. The immobilization parameters that affected apparent activity of immobilized lipase were confirmed in order to obtain the optimum conditions for the immobilized lipase on Mg–Al hydrotalcite and better understand the relationships between the immobilized variables (immobilization time, immobilization temperature, and enzyme/support ratio) and the response (relative lipase activity). Also, rape oil was utilized to synthesize biodiesel using immobilized lipases. Factors influencing synthetic utility of immobilized lipases were researched in synthesis of biodiesel such as molar ratio of methanol to oil, water content and free fatty acid in reaction system, and reuse times. The kinetics of thermal activation/deactivation of the immobilized and free lipases was also investigated. All the results obtained in this study would provide a sound basis for further exploration.

2. Materials and methods

2.1. Materials

Lipase powder (1100 U g^{-1}) from *S. cerevisiae* DX213, CCTCC NO: M 207082 stored in our laboratory and in the China Center for Type Culture Collection (CCTCC) was produced in a PDA medium at 30 °C. After 24 h of incubation, the medium was simply filtered. Supernatant was treated with ammonium sulphate (80% saturation). Then, the precipitate was dialyzed in water and lyophilized for using as a crude lipase preparation in powder form. Refined rape oil was purchased from the local supermarket. Chitosan (95% deacetylated degree, food grade) and silica gel (150–300 μm, analytical grade) were purchased from Ningbo Zhenhai Haixin Biological Products Co. Ltd. and Beijing Chemical Reagents Company, respectively. Gelatin was purchased from Shijiazhuang JinDa Gelatin Co. Ltd. γ-Al_2O_3 (≥99.99; specific surface area ≤260 m^2 g^{-1}) was purchased from Zhejiang Province Zhong Ming Chemical Science and Technology Limited Company. All other chemicals were of analytical grade.

2.2. Support synthesis and lipase immobilization

Mg–Al hydrotalcites were prepared by urea hydrolysis. The urea decomposition method was used to prepare Mg–Al hydrotalcites with a Mg/Al molar ratio of 4.0. $Mg(NO_3)_2{\cdot}6H_2O$ and $Al(NO_3)_3{\cdot}9H_2O$ were dissolved in deionized water which was added into a three-neck flask. Urea ([urea]/[NO_3^-] molar ratio of 4.0) was dissolved in the above solution. The flask was soaked in an oil bath previously heated at 105 °C to start the hydrolysis reaction. The pH was measured all along the reaction with an industrial pH electrode for high temperature (Mettler Toledo). The solutions were maintained at 105 °C for 10 h under stirring (300 rpm), and then were aged statically at the same temperature for another 18 h. The formed solids were collected by filtration and washed with deionized water and subsequently dried at 100 °C for 18 h. Then the samples were calcined at 500 °C for 7 h in a muffle furnace as a support.

Gelatin was immersed in aqueous solutions of 25 wt% glutaraldehyde for 24 h. The treated gels were dried at 25 °C in a vacuum oven for 12 h, and then refluxed in water using Soxhlets extractor for 24 h to remove the uncross-linked gelatin. Finally, the cross-linked gelatin was dried at 25 °C in a vacuum oven for 24 h.

The lipase powder was dissolved in the 0.1 mol L^{-1} phosphate buffer (pH 7.5) and the lipase activity in the lipase solution was adjusted to 720 U mL^{-1} before mixed with the supports. The supports were added into the lipase solution (720 U mL^{-1}) and stirred at 100 rpm for a period of time. The immobilized lipase was separated by filtration and washed with the buffer to remove the un-adsorbed soluble enzyme. The immobilized lipase was then lyophilized in freeze drier. The highest enzyme activity in each set of experiments was taken as maximum lipase activity. The relative activity at different set of experiments was determined compared to the maximum lipase activity.

$$\text{Relative activity}(\%) = \frac{\text{Lipase activity}}{\text{Maximum lipase activity}} \times 100$$

2.3. Determination of lipase activity

The activity of free and immobilized enzyme was assayed by titrating the fatty acid with 0.05 M NaOH. The fatty acid was liberated from the hydrolysis of olive oil under the catalysis of enzymes in 0.1 mol L^{-1} phosphate buffer (pH 7.5) at 37 °C. Activities were assayed by adding lipase in the buffer, using 5 mL 20% (v/v) olive oil emulsification solution as the substrate, which was obtained after pure olive oil

dispersed in water solution containing polyvinyl alcohol (4%, w/v). After exact 15 min of incubation at 37 °C, the reaction was stopped by adding 15.0 mL of alcohol solution (95%, w/w). Finally, the reaction solution was titrated with 0.05 M of NaOH. The blank hydrolysis of olive oil was a same process, except that the alcohol solution was added at the beginning of the hydrolysis. The fatty acid content was calculated from the difference between the blank and acid equation of the titration. One unit (U) of lipase activity was defined as the amount of enzyme that catalyzes the liberation of 1 μmol fatty acid from olive oil per min at pH 7.5 at 37 °C.

2.4. Kinetic parameters

The kinetic parameters, K_m and V_{max}, of free and immobilized lipase were calculated from the Lineweaver–Burk and Michaelis–Menten models using varying concentrations of olive oil emulsification solution as the substrate (as described in Section 2.2).

2.5. Enzymatic production of biodiesel

Refined rape oil (acid value 0.5 mg KOH g [oil]$^{-1}$; water content 0.2 wt%) with the immobilized lipase (1.5%, w/w of oil) were placed into a 500-mL three-angel necked flask equipped with reflux condenser and Teflon stirrer (100 rpm) under atmospheric pressure at 45 °C for 4.5 h, when a specific methanol was added slowly to the flask at the speed of 8 mL min^{-1}. After reaction, the methanol was recovered by a rotary evaporator in vacuum at 45 °C. Subsequently, the immobilized lipase was separated by filtration and the filtrate was allowed to settle overnight before removing the glycerol from the bottom in a separating funnel to get the ester layer on the top, called biodiesel.

2.6. Reusability

The immobilized enzyme was used in the repeated hydrolysis experiments at 45 °C. The experimental conditions were the same as described above with a molar ratio of methanol/oil of 4:1. After each cycle of the reaction, the immobilized enzymes were filtered, washed with the phosphate buffer (0.1 mol L^{-1}, pH 8.0) and dried at room temperature for 4 h. The immobilized enzymes were reused for further reaction.

2.7. Analytical methods

The moisture content of each rape oil used in this project was measured in triplicate as described by Dorado et al. [10]. Acid value was measured in triplicate according to the procedure of AOCS [11].

The biodiesel (methyl ester) was dried over sodium sulfate and analyzed by gas chromatography on a PerkinElmer GC-200 chromatograph with FID detector, equipped with a stainless steel packed column (3 mm × 2 m Silar-9cp). The oven temperature program consisted of: start at 160 °C (2 min), ramp at 1.5 °C min^{-1} to 215 °C (10 min). Undecanoic acid methyl ester was used as the internal standard. The formed methyl esters were identified by comparing their retention times to the standard retention times of fatty acid methyl esters. The concentration of methyl ester was used to calculate the ester conversion of rape oil into biodiesel. The ester conversion (%) was calculated by the following equation,

$$\text{Ester conversion}(\%) = \frac{m_{\text{actual}}}{m_{\text{theoretical}}} \times 100$$

where both m_{actual} (g) and $m_{\text{theoretical}}$ (g) were the mass of methyl esters obtained actually and theoretically, respectively.

2.8. Statistical analysis

All the experiments were carried out three times in order to determine the variability of the results and to assess the experimental errors. In this way, the arithmetical averages and the standard deviations were calculated for all the results.

3. Results and discussion

3.1. Immobilization of lipase

3.1.1. Support comparison

There were different materials used for immobilization of lipase. However, the widely different substrates and supports employed led to an enormous amount of quantitative data, which cannot be directly compared. So, the effect of immobilization material on the lipase immobilization was investigated (Fig. 1A). The results showed that the highest apparent lipase activity of 725 U g^{-1} was on the hydrotalcite with a Mg/Al molar ratio of 4.0, followed by 670 U g^{-1} on γ-Al_2O_3 and the lowest lipase activity was 565 U g^{-1} on cross-linked gelatin. Mg–Al hydrotalcite was more efficient as a support for *S. cerevisiae* lipase as compared to other supports in the experiment.

The hydrotalcites with Mg–Al molar ratios of 2.0, 3.0, 4.0, and 5.0 were used as supports to immobilize the lipase (Fig. 1B). The relative lipase activity for 6 h adsorption was improved from 63.72% to 100%, when the molar ratios of Mg/Al were increased from 2.0 to 4.0. When the Mg/Al molar ratio was 5.0 (greater than 4.0), the relative activity decreased to 86.21%. As a result, the relative activity of the immobilized lipase on the Mg–Al hydrotalcite at ratio 4.0 showed a maximum, which was due to better adsorption of protein such as better porosity and large surface area and the base strength [12,13]. A similar result was also reported by others [12]. Thus, hydrotalcite with a Mg/Al molar ratio of 4.0 was used for further work.

3.1.2. Effect of amount of support

The amount of lipase immobilized on a carrier was limited because the porous sites as the carriers were saturated [14]. In order to find the optimal amount of the lipase, we tested the immobilization of various support amounts in the lipase solution (720 U mL^{-1}) for 6 h adsorption. The effect of support amounts on relative activity was shown in Fig. 2. The relative activity significantly increased when support amount was increased from 0.16 to 0.32 g mL^{-1}, then decreased above 0.32 g mL^{-1}. The highest relative activity was achieved when support amount was 0.32 g mL^{-1}. At a higher support amount (above 0.32 g mL^{-1}), the lipase molecules seemed to maximize the contact with the surface of support, which may result in a loss of lipase conformation and, consequently in reduced activities. However, for a support amount lower than 0.32 g mL^{-1}, multilayer adsorption might have occurred and effectively inhibited access to the enzyme active sites [15].

3.1.3. Effect of immobilizing time

The duration of enzyme immobilization would affect the amount of immobilized enzyme on the hydrotalcite. So, the immobilization time from 2 to 14 h was also investigated and the relative activities of the lipase immobilized on the hydrotalcite were measured (Fig. 3). It was found in 4 h, the relative activity reached about 93.0% of the maximum, and relative activity increased rapidly with increasing immobilization time periods from 2 to 6 h because the amount of immobilized lipase on the Mg–Al hydrotalcite increased. But above the immobilization time of 6 h, relative activity declined gradually, probably because the long-term agglomeration of the lipase in the interlayer or porosity of the hydrotalcite would result in degradation of the lipase activity. Similar phenomenon was reported with lipase immobilization by physical adsorption [15]. Thus, 6 h of lipase immobilization time was used throughout our studies.

3.1.4. Effect of pH and temperature

The choice of pH for the enzyme immobilization must been taken into consideration. In the optimum pH required for adsorption and the pH range over, a particular enzyme was stable, or its activity loss was negligible. To get the optimum pH for immobilization of the lipase on hydrotalcite, pH of the lipase solution was changed between 5.0 and 9.0 at 30 °C for 6 h. As seen in Fig. 4A, the relative activity of the immobilized lipase was affected from the lipase solution pH. The maximum relative activity occurred at pH 7.5. At pH greater than 7.5, the relative activity was speedily decreased with further increasing pH up to 9.0. The pH had an effect on the ionic state of the lipase molecules as well as the polarity of the ionic groups on the surface of the hydrotalcite. At pH 7.5, the enzymatic polarity might be weakened, which could enhance the lipase binding onto the hydrotalcite surface.

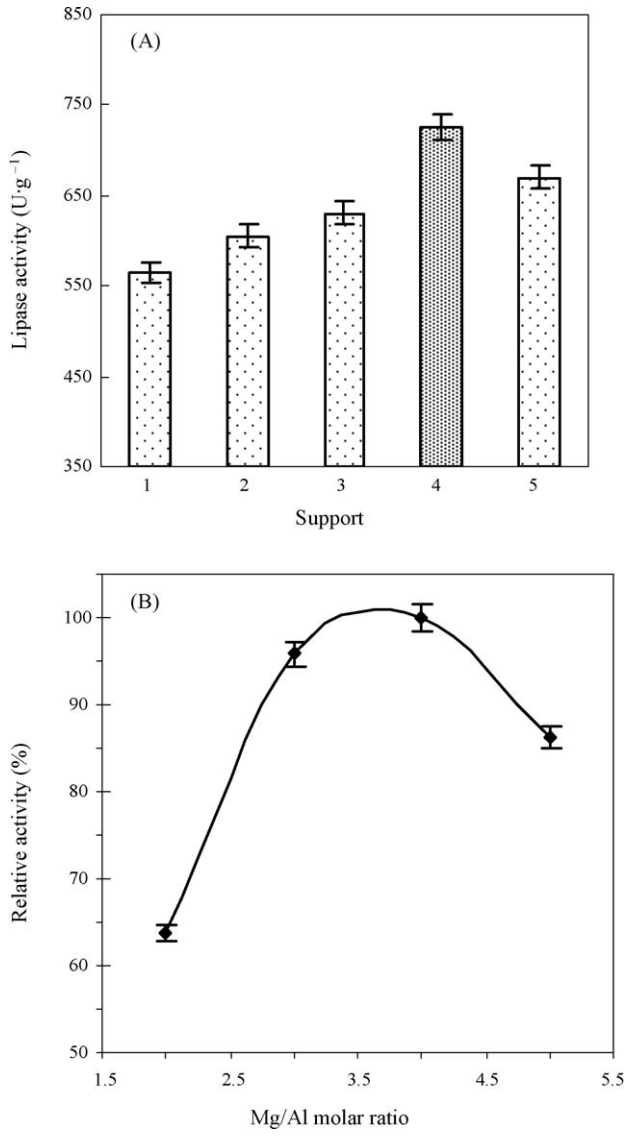

Fig. 1. Activity of immobilized lipases on different supports, performed at pH 7.5, a loading of 0.32 g mL^{-1} support, and 6 h adsorption at 30 °C. (A) 1: cross-linked gelatin; 2: silica gel; 3: chitosan; 4: Mg–Al hydrotalcite with a Mg/Al molar ratio of 4.0; 5: γ-Al_2O_3. (B) Mg–Al hydrotalcite with different Mg/Al molar ratios.

Lipase was immobilized on hydrotalcite at different temperatures between 25 and 80 °C at pH 7.5 for 6 h. The results in Fig. 4B indicated that the optimum temperature of lipase immobilization was 30 °C, and the relative activity was near its maximum value in the range of 30–35 °C. In practice, immobilization temperature can be controlled at 30–35 °C. Temperatures that were too high may cause the thermal deactivation of the lipase.

3.2. Characterization of free and immobilized lipase

The effect of pH and temperature on enzyme activity was determined by incubating free and immobilized lipase separately at different pHs and temperatures for 30 min. At the end of incubation time the residual lipase activity of each sample was determined.

3.2.1. Effect of pH and temperature on free and immobilized lipase

Effect of pH on the activities of free and immobilized lipases was investigated in the pH range 5.0–9.0 at 35 °C and the results were given in Fig. 5A. It was observed that the optimum pH of free lipase

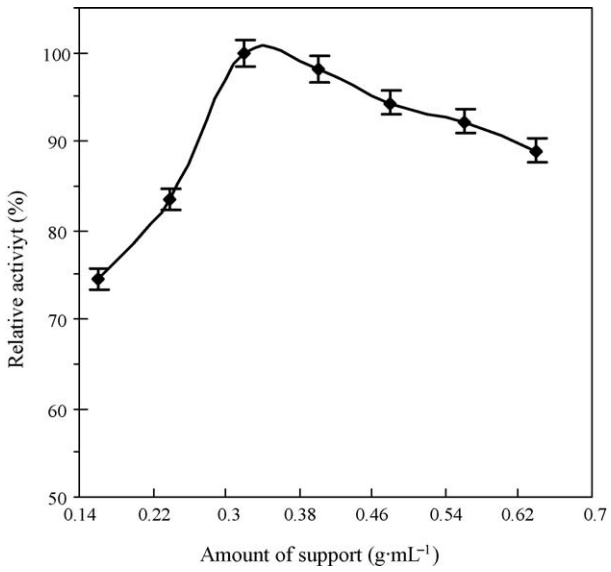

Fig. 2. Effect of amount of support on lipase immobilization, performed at pH 7.5 and 30 °C for 6 h.

was 7.5 while it was shifted to 8.0 for the immobilized lipase. We were expecting a considerable shift in optimal pH of immobilized lipase to basic region due to the anion support which may cause a decrease in the pH of the microenvironment of the immobilized enzyme, for Mg–Al hydrotalcite was an anion support [16]. Furthermore, it was observed that the immobilized lipase was less affected from the medium pH than the free lipase.

The effect of temperature on the activity of free and immobilized lipase at pH 7.5 for the free lipase and at pH 8.0 for the immobilized lipase was shown in Fig. 5B. The optimum temperatures for free and immobilized lipases were 40 and 45 °C, respectively. As the case of pH effect, the immobilized lipase had a broader tolerance range to heat than the free lipase. The relative activities of the free and immobilized lipases remained over 85% and 90% in the temperature range 30–55 °C, respectively. The immobilized lipase was found to retain its lipase activity at a higher temperature compared with the free lipase. At 50 and 60 °C, the free lipase expressed 88.46% and 75.38% retention of relative activity, respectively, whereas the immobilized lipase expressed

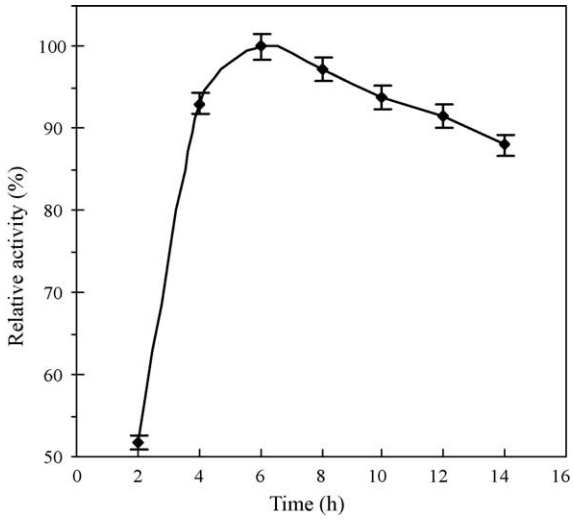

Fig. 3. Effect of immobilization time on lipase immobilization, performed at pH 7.5, a loading of 0.32 g mL^{-1} support, and 30 °C.

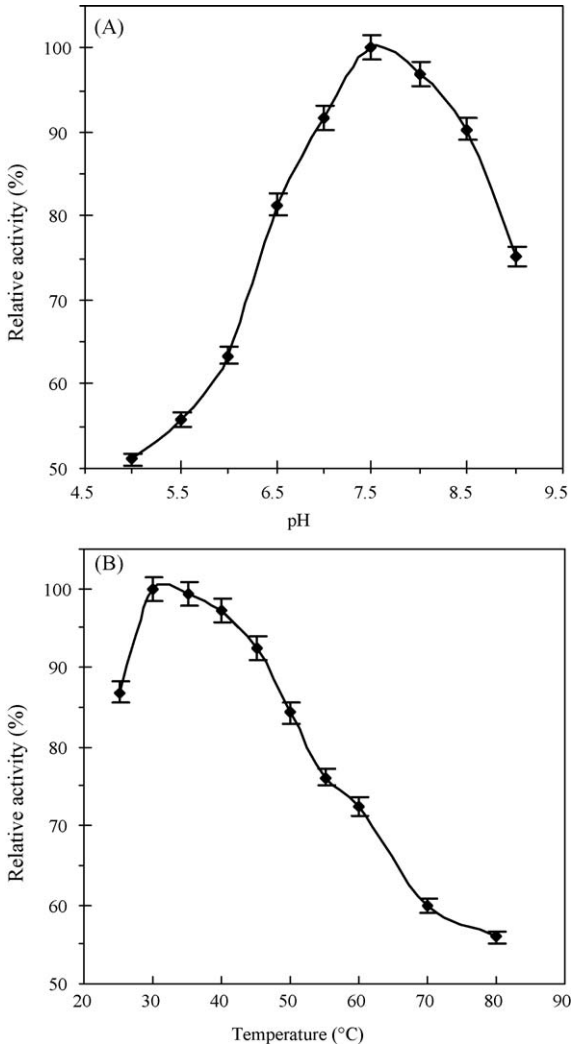

Fig. 4. Effect of pH (A) and temperature (B) on lipase immobilization with a 0.32 g mL^{-1} support for 6 h.

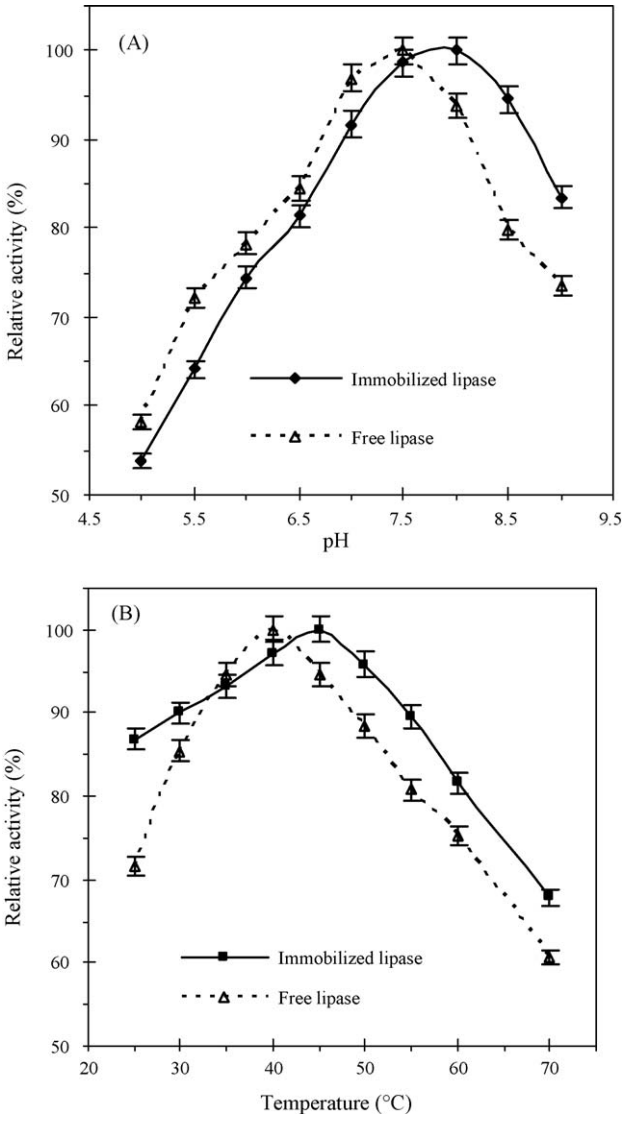

Fig. 5. Effect of pH (A) and temperature (B) on activity of free and immobilized lipase with a 0.32 g mL^{-1} support.

95.86% and 81.62% retention, respectively. The activity of immobilized lipase was observed to be more thermostable than that of free lipase. A similar result was also obtained in the analyses of kinetics of thermal activation/deactivation of the immobilized and free lipases (Fig. 7B). The process of immobilization generally offered the microenvironment for lipase and tended to minimize the effect of external factors such as temperature, pH and ionic species. At higher temperature, soluble enzyme easily underwent denaturation while immobilized enzyme was protected probably in terms of rigid conformation and therefore, was able to retain its catalytic activity [17].

3.2.2. Kinetic constants

Comparison of the K_m value for a given free and immobilized enzyme could provide information about interaction between enzyme and its support [17]. The effect of substrate concentration on the initial reaction rate (V) catalyzed by the free and immobilized lipases was studied using olive oil as the substrate (Fig. 6). Experiments were conducted at predetermined optimal conditions. From Lineweaver–Burk and Michaelis–Menten models, Michaelis constant (K_m) and the maximum reaction rate (V_{max}) of the free and immobilized lipases were calculated and the results were given in Fig. 6. The values obtained for V_{max} were 4.1017 and 2.8952 mg min^{-1} and the apparent K_m were 4.3437 and 5.4470 mg mL^{-1} for the free and immobilized lipases, respectively. The K_m value for the immobilized lipase was 1.3 times higher than that for the free lipase. An increase in K_m once an enzyme had been immobilized, indicated that the immobilized enzyme had an apparent lower affinity for its substrate than that of free enzyme does, which might be caused by the steric hindrance of the active site by the support, the loss of enzyme flexibility necessary for substrate binding, or diffusional resistance to solute transport near the particles of the support [17]. The result showed that the affinity of immobilized lipase for olive oil was smaller than that of the free lipase, namely, the activity of immobilized lipase lower than that of the free lipase. The change of kinetic constants may be a consequence of either the structural changes in the enzyme introduced by the immobilization procedure or lower accessibility of substrate to the active sites of the immobilized enzyme [17]. The V_{max} value of the immobilized enzyme was therefore smaller than that of the free enzyme.

3.3. Thermodynamic parameters

3.3.1. Activation energy of free and immobilized lipases

The relative activities of the free and immobilized lipases increased with increasing temperature from 25 °C to 40 and 45 °C,

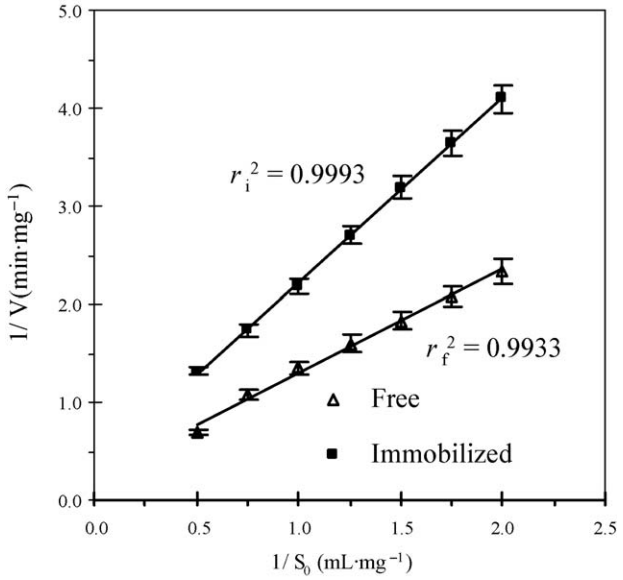

Fig. 6. Kinetic parameters of the immobilized and free lipases.

respectively (Fig. 5B). Thermal activation of the lipases, in free and immobilized forms, can be simply described by the Arrhenius equation [18,19]:

$$\mathrm{AT} = A \exp\left(\frac{-E_a}{RT}\right) \quad (1)$$

where AT was enzyme activity, A was the pre-exponential factor, E_a was the activation energy, R was the gas constant and T was the absolute temperature in Kelvin. The dependence of lipase activity on temperature was observed to follow an Arrhenius type reaction for the free and immobilized lipases in temperature ranges of 25 °C to 40 and 45 °C, respectively. The data were plotted ln AT versus the reciprocal of the absolute temperature ($1/T$) to give a straight line with a slope of $-E_a/R$ (Fig. 7A). The activation energy values of the free and immobilized were calculated as 6.96 and 2.42 kJ mol^{-1} with the correlation coefficient of $r_f^2 = 0.9434$ and $r_i^2 = 0.9836$, respectively. It was evident that the value of E_a with the immobilized lipase was significantly lower than that of the free lipase, meaning that immobilized lipase was insensitive to temperature.

3.3.2. Thermal deactivation of free and immobilized lipases

In order to assess the enzyme stability, the loss of enzyme activity should be investigated. Under the optimal conditions, the main reason for the loss of enzyme activity was thermal deactivation of the enzyme [20]. It was generally accepted that the thermal deactivation of enzyme was a first-order reaction. Therefore, the following enzyme deactivation rate model could be obtained following an Arrhenius type reaction [17,20].

$$\mathrm{Ln\,DAT} = \frac{-E_d}{RT} + \mathrm{ln\,DA} \quad (2)$$

where DAT was the enzyme activity in deactivation, DA was the initial enzyme activity in deactivation and E_d was the inactivation energy. The relative activities of the free and immobilized lipases decreased with increasing temperature from 40 and 45 to 70 °C, respectively (Fig. 5B). The dependence of lipase activity on temperature was observed for the free and immobilized lipases in temperature ranges of 40 and 45–70 °C, respectively. The value of E_d for the immobilized lipase, 6.27 kJ mol^{-1} ($r_f^2 = 0.9793$), was lower than that for the free lipase (6.51 kJ mol^{-1}, $r_i^2 = 0.9704$),

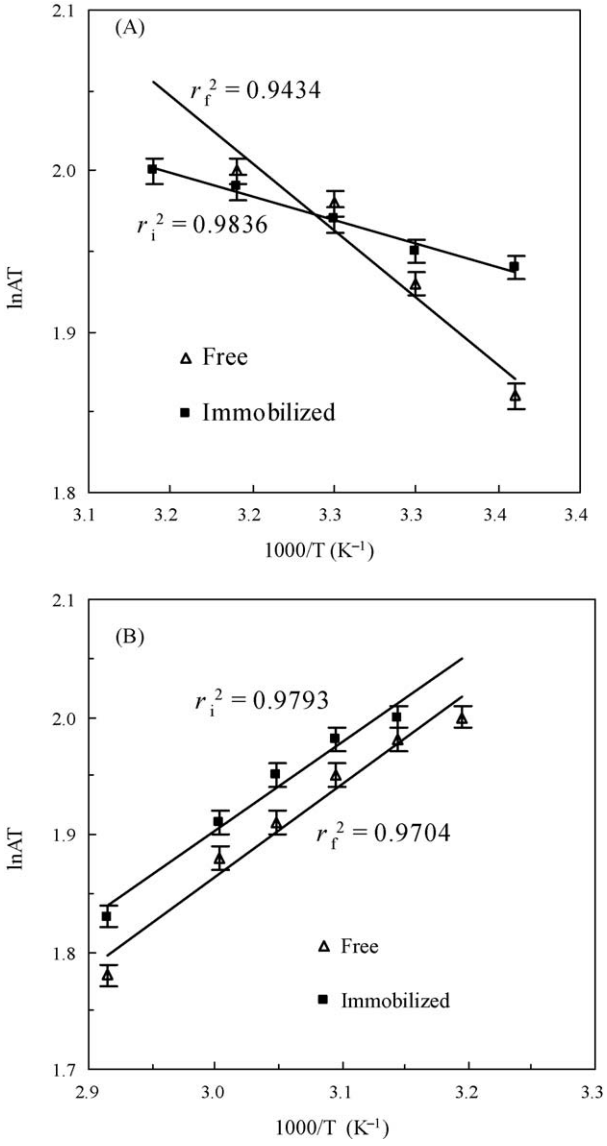

Fig. 7. Thermal activation of the free and immobilized lipases. (A) Activation energy of free and immobilized lipases; (B) deactivation energy of free and immobilized lipases.

implying the immobilized lipase was less temperature-sensitive and harder to deactivate [17].

3.4. Production of biodiesel using immobilized lipase

3.4.1. Effect of molar ratio of methanol to oil

The stoichiometric amount of ethanol had to be used in order to achieve total conversion of triglycerides into their ethyl esters. The gradual addition of methanol (8 mL min^{-1}) was conducted in order to avoid the lipase deactivation by a high initial alcohol concentration. The effect of methanol/oil molar ratios from 3:1 to 8:1 were evaluated in the transesterification reaction conducted at 45 °C with 1.5% immobilized lipase (by weight of oil) for 4.5 h (Fig. 8). The results showed that methanol/oil molar ratio of 4 allowed the highest ester conversion (96.5%). The lipase activity and ester conversion at higher methanol/oil molar ratio from 4:1 to 8:1 decreased with increase in methanol/oil molar ratios. Similar results were observed for the methanolysis of vegetable oils using lipases [21]. The decreased activity and ester conversion at higher methanol concentrations were mainly because of the methanol

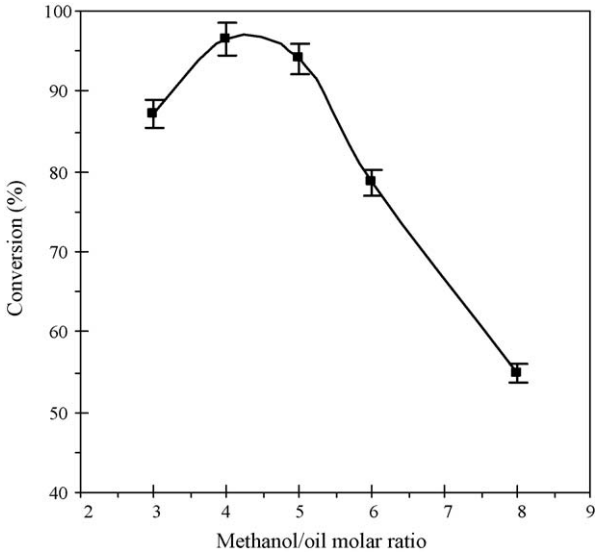

Fig. 8. Effect of methanol/oil molar ratio on ester conversion.

excess to distort the essential water layer that stabilized the immobilized enzyme [22]. So, a 4:1 molar ratio of methanol to oil was used for further biodiesel production.

3.4.2. Effect of water content and free acid fatty content in oil

Lipases possessed the unique feature of acting at the interface between an aqueous and an organic phase. Lipase interfacial action was due to the fact that their catalytic activity generally depended on the aggregation of the substrates. Activation of the enzyme involved unmasking and restructuring of the active site through conformational changes of the lipase molecule, which required the presence of oil–water interface. Lipase activity generally depended on the available interfacial area. With the increased addition of water, the amount of water available for oil to form oil–water droplets increased; thereby increased the available interfacial area. However, since lipases usually catalyzed hydrolysis in aqueous media, excess water might also stimulate the competing hydrolysis reaction. The optimum water content was a compromise between minimizing hydrolysis and maximizing enzyme activity for the transesterification reaction [2]. In order to study the effect of water content present in the reaction mixture on methanolysis, the water content varied from 0.2 to 3.5 wt% of rape oil with molar ratio of methanol/oil of 4:1 at 45 °C for 4.5 h (Fig. 9A). Lipase could show the maximal activity (96.4%) with an appropriate water content (2.0 wt%). As can be seen in Fig. 9A, the activity increased with increasing water content in the range from 0.2 to 2.0 wt% of oil, and then decreased slightly when further increasing water content (above 2.0 wt%). High water activity will favor hydrolysis, whereas a low water activity will favor esterification [2,23].

Refined rape oil with 0.5 mg KOH g $[oil]^{-1}$ acid value was adjusted with oleic acid from 0.5 to 6.0 mg KOH g $[oil]^{-1}$ of acid value. Under the same reacting conditions, the effect of acid value on ester conversion catalyzed by the immobilized lipase was shown in Fig. 9B. The ester conversion declined with increase in acid value. The acid value of less than 3.5 mg KOH g $[oil]^{-1}$ resulted in ester conversion above 81.7% after 4.5 h reaction. Up to an acid value 3.5 mg KOH g $[oil]^{-1}$, the ester conversion decreased rapidly. With acid value 4.0 mg KOH g $[oil]^{-1}$, the ester conversion was much lower and only attained 66.9% for 4.5 h reaction. So, successful catalytic transesterification was obtained at acid value less than 3.5 mg KOH g $[oil]^{-1}$. It was markedly lower than the acid value in the waste oil catalyzed by the commercial lipases reported

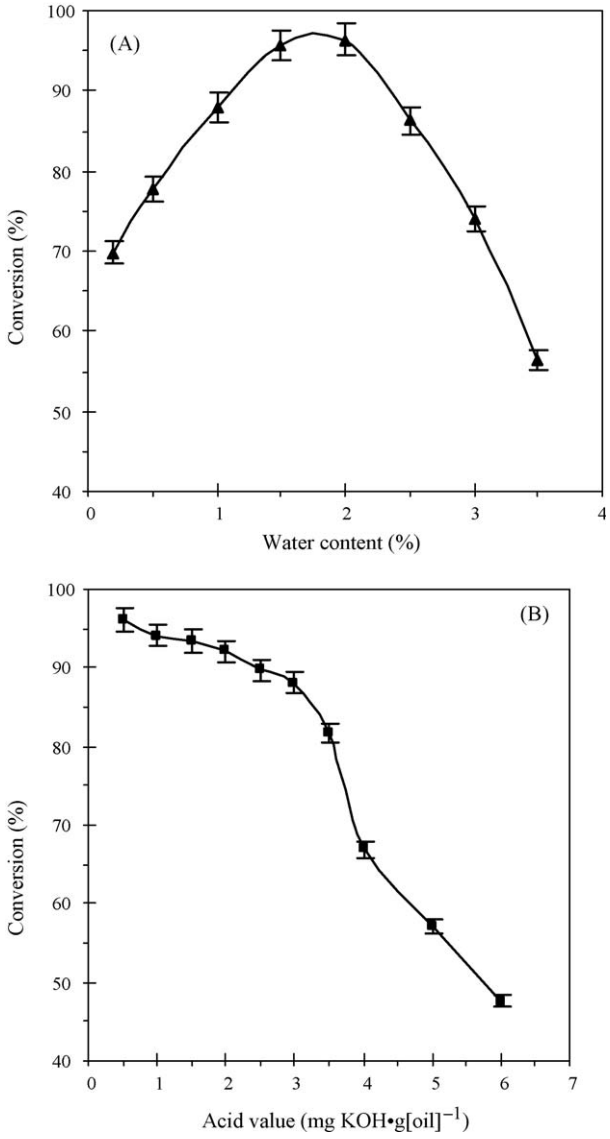

Fig. 9. Effect of water content (A) and acid value (B) in rape oil on ester conversion.

in the literature [24]. The result showed that the immobilized lipase from *S. cerevisiae* was sensitive to free fatty acid in rape oil.

3.4.3. Effect of reusability

The duration of a catalyst was an important feature for its potential application in industry. Operational stability or reusability was of high importance in determining immobilized enzyme efficiency. To investigate the reusability of immobilized lipase, 15 repetitive uses of immobilized lipase were operated (Fig. 10). Fig. 10 showed the profile of immobilized lipase reusability for the transesterification with reaction time 4.5 h in each cycle. High ester conversion was maintained at above 81% from cycle 1 to 10 for the immobilized lipase. However, the ester conversion was only 66.7% in the 13th cycle, indicating that the immobilized lipase activity started to decrease rapidly thereafter from cycle 13 and 14. The ester conversion retained approximately 54.1% at the end of 14 uses. The decrease in the ester conversion after several runs was due to the turnover of large quantities of substrates that resulted in the production of substantial quantities of water (as co-product), the denaturation of the lipase and loss of lipase during filtration since no make-up quantities were added [12].

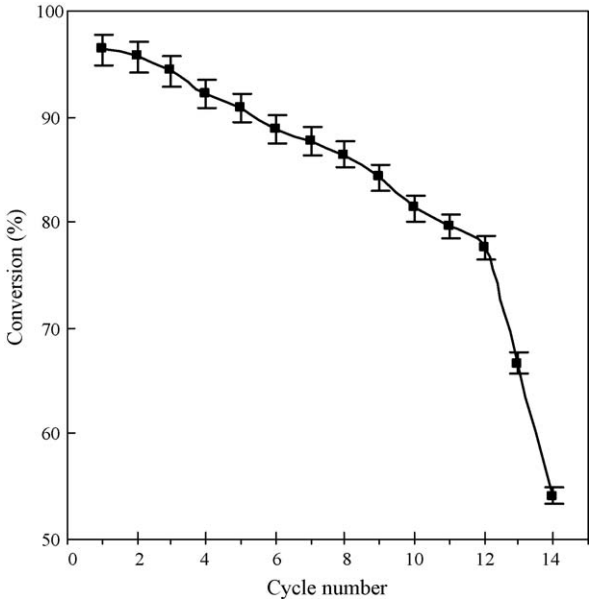

Fig. 10. Stability of the immobilized lipase for repeated cycles.

From this study, immobilization of lipase onto Mg–Al hydrotalcite through physical adsorption has been proven to be a useful technique for improving enzyme activity. The result showed that the immobilized lipase on Mg–Al hydrotalcite had a good durability.

4. Conclusions

Enzymatic transesterification of vegetable oils offered an environmentally more attractive option to the conventional physiochemical process. Approach of physical adsorption technique for the immobilization was relatively easy and inexpensive. An effective mean for achieving the biodiesel of methyl ester using immobilized Mg–Al hydrotalcite–lipase as a biocatalyst have been achieved. Mg–Al hydrotalcite seemed to be a well-suited support for the immobilization of lipase from *S. cerevisiae* for the transesterification of rape oil with methanol. The lipase had outstanding performance in relation to the transesterification activity, ester converting about 96% in 4.5 h. The properties and stability of the immobilized lipase exhibited interesting characteristics that may be suitable for industrial biotransformations. The present work demonstrated a promising application potential of the Mg–Al hydrotalcite for enzyme immobilization.

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