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Biodiesel production by enzymatic transesterification catalyzed by *Burkholderia* lipase immobilized on hydrophobic magnetic particles

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HIGHLIGHTS

- *Burkholderia* lipase was successfully immobilized on hydrophobic magnetic particles.
- The immobilized lipase could be repeatedly used six times without severe activity loss.
- Biodiesel conversion catalyzed by immobilized lipase reached nearly 70% within 12 h.
- The transesterification conditions with the immobilized lipase was optimized by RSM.

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ABSTRACT

Biodiesel is a promising substitute for petroleum diesel, and has been commercialized and utilized in many countries. Conventional chemical or physical methods used for biodiesel production face the drawbacks of high energy consumption or intensive use of chemicals. In contrast, using lipase-catalyzed transesterification for biodiesel synthesis is clean, effective, and water tolerance. Therefore, in this work, a self-developed *Burkholderia* lipase was immobilized onto hydrophobic magnetic particles (HMPs) for biodiesel production. Transesterification with the immobilized lipase could be repeatedly carried out six times without severe activity loss. The optimal conditions for the enzymatic transesterification were identified as: room temperature, 200 rpm agitation, 10% water content, and a methanol-to-oil molar ratio of 4:1. Under these conditions, the conversion of oil to fatty acid methyl esters (FAMEs) reached nearly 70% within 12 h, giving a biodiesel production rate of 43.5 g/L/h.

1. Introduction

Due to the rise in demand for energy and the limited supplies of fossil fuel available, more attention has recently been focused on developing clean and renewable sources of alternative energy [1,2]. Among the possible candidates for an alternative energy source, biodiesel has attracted much attention because its physical and chemical properties and energy content are similar to those of petroleum diesel [3]. In addition, the emission of particulates, CO, and unburned hydrocarbons from biodiesel combustion in a diesel engine are all lower than that those from petroleum diesel [4–6]. Biodiesel could be produced by transesterification of triglycerides. Transesterification is conventionally performed by alkaline or acid catalysis, which is restricted by impurities such as water and free fatty acids [7–10]. Another method to produce biodiesel is to use supercritical reactions, although these are usually performed at high temperature and pressure, thus requiring significant energy consumption and high operating costs. A last method is to utilize enzymatic reactions by using lipase. Lipase can perform the esterification of free fatty acids and transesterification of triglycerides without the formation of soaps. The main products when using lipase as the catalyst in transesterification are biodiesel and glycerol, resulting in the easy removal of lipase [11,12], which is an important issue when choosing a catalyst.

The major barrier to the wider use of enzymatic transesterification is the cost of lipases, but the enzyme cost can be reduced by the enzyme immobilization, which enables repeated uses of the enzyme [13]. Moreover, the stability, activity, and reusability of

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enzymes can also be improved by immobilization [14]. Literature shows that lipases strongly adsorb on the hydrophobic interfaces by their lids and protein chains [15,16]. For this reason, hydrophobic interaction has became the most popular method for lipase immobilization, and various hydrophobic materials have been used to achieve this, such as Accurel EP-100, Octyl agarose, PSLG-modified PPMM, and polysulfone nanofibrous membranes [17–20]. However, better immobilized lipase systems with characteristics of being more effective, more durable, and less expensive are still of great demand to facilitate the commercialization of enzymatic biodiesel synthesis processes.

Therefore, in this work, the lipase produced by an isolated *Burkholderia* sp. strain [21–23] was immobilized onto magnetic particles by hydrophobic adsorption approaches. The immobilized lipase was characterized in terms of its enzymatic activity and the reusability. The conditions for transesterification catalyzed by the immobilized lipase were also optimized to assess the feasibility of using the developed immobilized lipase in practical applications.

2. Materials and methods

2.1. Strains and cultivation

The lipase-producing strain was identified as *Burkholderia* sp. which was isolated from food wastes in central Taiwan [22]. The culture medium consisted of (per L): olive oil, 10 mL; yeast extract, 2.175 g; $(NH_4)_2SO_4$, 6 g; HEPES (4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid), 2.4 g; KCl, 4.45 g; $CaCl_2$, 0.02 g; hexadecane, 10 mL; and $MgCl_2$, 0.2 g. *Burkholderia* sp. was cultivated in a 5 L fermentor at 30 °C and 400 rpm for 30 h for the production of lipase [21–23].

2.2. Preparation of hydrophobic magnetic particles (HMPs)

Two hundred milliliters 8.85% $FeSO_4$ solution was mixed with 100 mL KNO_3 prior to heating to 90 °C. After this, 27.62% KOH 50 mL was added to the solution before incubating at 90 °C for 2 h. The magnetic particles collected from the solution were washed by osmosis water until the pH of the decanted water became neutral. Magnetic particles were then covered with a silica layer by silication. The mixture for silication was 10 g magnetic particles, 30 mL TEOS (tetraethoxysilane), 80 mL ethanol, 24 mL osmosis water, and 28 mL NH_4OH. The solution was kept at room temperature for 12 h. HMP was prepared by the following steps. 15 g silicated magnetic particles were mixed with 150 mL ethanol and 150 mL glycerol prior to incubating at 85 °C. After that, 10 mL [3-(trimethoxysilyl) propyl] octadecyldimethylammonium chloride was added to the solution. After 2 h, the resulting particles were collected prior to being dried.

2.3. Adsorption curve of lipase on HMP

A 0.55 g HMP was placed into the 50 mL broth containing 25 U/mL crude lipase. The conditions were room temperature and 600 rpm with the solution pH controlled at 6.5. The residual activity of the lipase in the mixture was determined at 2, 4, 8, or 24 h.

2.4. Langmuir isotherm of lipase adsorption on HMP

Forty milliliters crude enzyme solution with the initial lipase activity of 3–40 U/mL was prepared in advance. 0.048 g HMP was placed into the enzyme solution with pH controlled at 6.5 at room temperature and 600 rpm. After 4 h, the lipase activity of the solution and immobilized enzyme were determined to obtain the Langmuir isothermal adsorption curve.

2.5. Immobilization of lipase

In our previous work, the *Burkholderia* lipase was immobilized by membrane [24] and Celite carriers [25], but the transesterification activity of using the two types of immobilized lipase was quite low. Thus, in this study, a new approach was used for lipase immobilization. The immobilization procedures are described as follows. In general, 15 g of HMP was added to the 2 L culture supernatant at room temperature. The pH of the mixture was controlled at 6.5. After 8 h, the immobilized lipase (HMP-lipase) was harvested prior to washing with 10 mM HEPES solution several times. After that, the particles were lyophilized at −55 °C and 10 Pa for 24 h. The resulting particles were stored at −20 °C before they were used.

2.6. Measurement of hydrolytic activity of lipase

Fifteen milliliters emulsified substrate (10 mL olive oil and 5 g gum arabic mixed with 100 mL osmosis water) was diluted with 35 mL osmosis water. HMP-lipase and the diluted solution were placed in a 100 mL vial prior to the analysis. The free fatty acid produced from the hydrolysis of olive oil was titrated by 0.1 N NaOH at pH 9.0 and 55 °C.

2.7. Transesterification catalyzed by the immobilized lipase

Ten milliliters olive oil (ca. 9.1 g), 1.66 mL methanol (ca. 1.33 g), and 1.5 mL water were mixed with HMP-lipase of 300 U to perform transesterification. The initial condition was methanol to oil in the molar ratio of 4 to 1, 30 °C, and 600 rpm. For the optimization of transesterification, the temperature of 20–60 °C, agitation rate of 200–650 rpm, water content of 5–40%, and methanol concentration of 0–36% were investigated. For the stepwise addition experiments, methanol was added into the solution with the methanol to oil molar ratio of 1.33 to 1 at the 6th hour, 10th hour, and at the beginning of the reaction.

2.8. Estimation of the conversion of transesterification

A gas chromatograph equipped with a Rtx-Biodiesel TG column and flame ionization detector (FID) was used to analyze the products resulting from lipase-catalyzed transesterification of olive oil with methanol. Methyl oleate and methyl palmitate were used as the standards. The temperature for injection and FID was 350 °C. The temperature of the column was initially maintained at 80 °C for 5 min. Following this, the temperature was increased to 380 °C at the rate of 15 °C/min and kept on 380 °C for 5 min. The conversion of transesterification occurred based on the following equation:

$$\text{Conversion}(\%) = \frac{\text{The concentration of FAME}}{\text{The concentration of FAME obtained from chemical catalysis}}$$

2.9. Reusability of HMP-lipase

The HMP-lipase was repeatedly used when the transesterification was conducted using a methanol-to-oil molar ratio of 4 to 1. Before the addition of 1000 U HMP-lipase, 9.1 g olive oil, 1.33 g methanol, and 1 mL RO water were mixed. The conditions were 30 °C and 600 rpm. When the conversion of olive oil achieved 60%, HMP-lipase was harvested from the solution by magnets. Fresh reagents were subsequently mixed with the harvested HMP-lipase for the next experiment under the same conditions.

3. Results and discussion

3.1. Langmuir adsorption isotherm of immobilized lipase

In order to find out the adsorption capacity of HMP (hydrophobic magnetic particles) for the lipase, the Langmuir adsorption isotherm (Eq. (3.1)) was employed, as follows:

$$q = \frac{q_{\max} C_e}{K_d + C_e} \tag{3.1}$$

where q is the adsorption capacity (U/g), C_e is the equilibrium concentration of protein (U/mL), $q_{\max}$ is the maximum adsorption capacity (U/g) and K_d is the dissociation constant. Based on the result of the regression analysis, the maximum lipase adsorption capacity of HMP is 4619 U/g, and the dissociation constant is 2.16 U/mL (Fig. 1a). This indicates that HMP has a promising affinity for the lipase due to its high adsorption capacity to lipase. Fig. 1a also shows that the experimental data fit perfectly to the Langmuir isotherm model. Moreover, the adsorption dynamics results (Fig. 1b) show rapid adsorption of the lipase on the hydrophobic group (i.e., HMP) for the first 3 h, after which the adsorption began to slow down and gradually reached saturation. The initial adsorption rate of lipase on HMP was 6250 U/h/L when initial lipase concentration was 25 U/mL and loading of the HMP matrix was 11 g/L.

3.2. Characterization of immobilized lipase (HMP-lipase)

As indicated in Fig. 2a, the production of FAME (fatty acid methyl ester) by free lipase was slightly higher than that by HMP-lipase. It is likely that the mass transfer resistance of methanol moving to immobilized lipase might increase due to the retention of oil on the hydrophobic surface of HMP. This increase in mass transfer resistance led to the lower transesterification rate, thereby leading to lower FAME production. Although the free lipase had slightly higher activity, the high enzyme cost hinders the commercialization of the lipase-catalyzed biodiesel synthesis process as the free lipase cannot be used repeatedly. One way of reducing the enzyme cost is to enhance the stability and/or reusability of lipase by immobilization technology to allow easy reutilization of the lipase. Therefore, the reusability of HMP-lipase was also examined in this work. The results (Fig. 2b) show that HMP-lipase can be repeatedly used for six runs of transesterification without the loss of its activity. The conversion of olive oil can still reach 60% within 12 h at the 6th batch run. The transesterification by HMP-lipase was also compared with that by a commercial lipase Novozyme 435 [26] as indicated in Table 1. It shows that the FAME production rate of HMP-lipase (42.1 g/L/h) and Novozyme 435 (41.2 g/L/h) are quite similar, but the cost for HMP-lipase (1.07 US$/kU) is lower than that for Novozyme 435 (3.2 US$/kU).

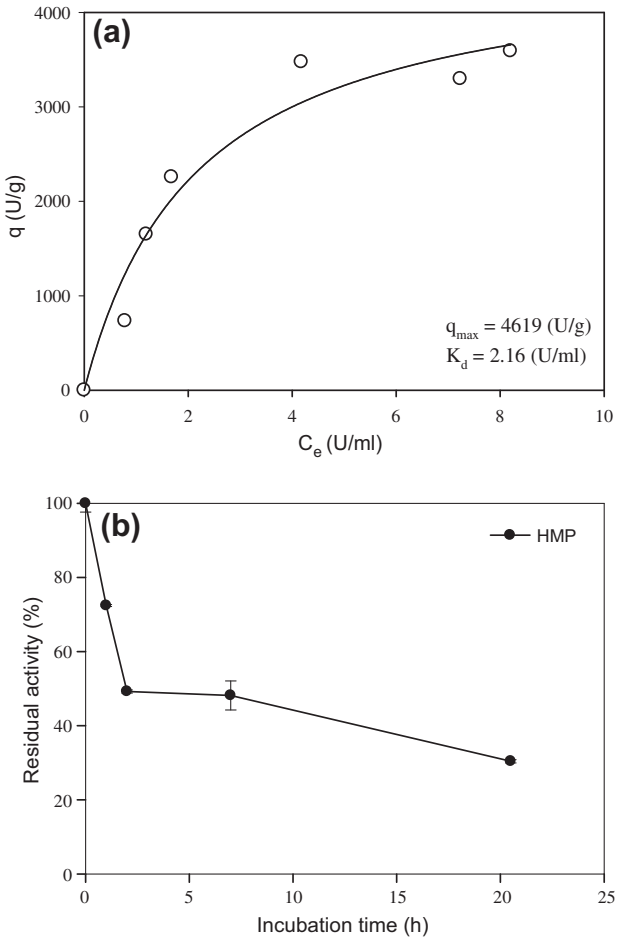

Fig. 1. Adsorption of *Burkholderia* lipase on HMP matrix. (a) Langmuir isotherm (lipase loading: 3–40 U/mL; HMP loading: 1.2 g/L) and (b) time-course adsorption profiles (lipase loading: 25 U/mL; HMP loading: 11 g/L). The operating temperature was 30 °C).

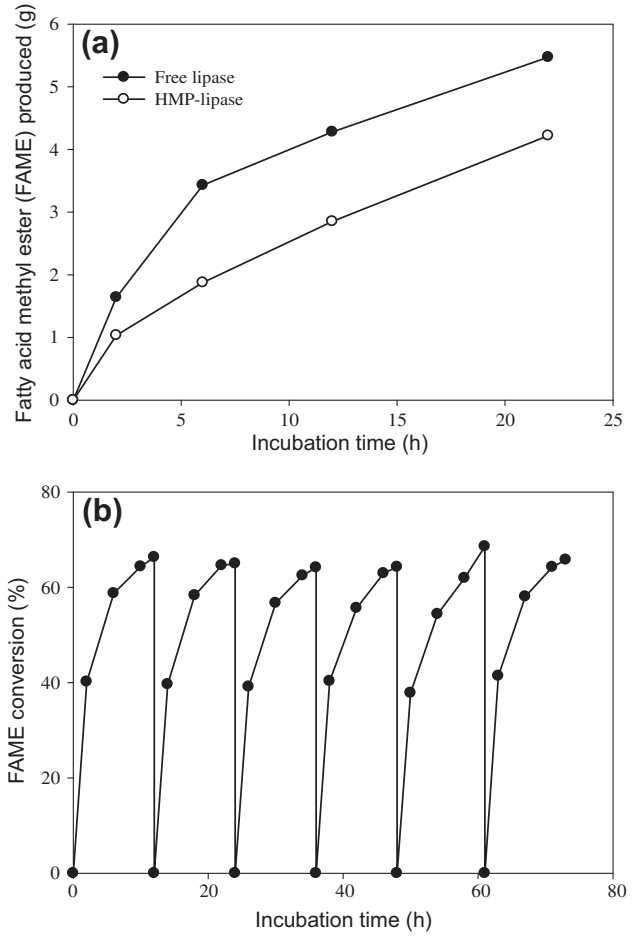

Fig. 2. (a) Production of fatty acid methyl ester (FAME) via transesterification of olive oil with methanol catalyzed by free and HMP-immobilized lipase and (b) the reusability of HMP-immobilized lipase in the transesterification reaction (lipase loading: 300 U/mL; methanol-to-oil molar ratio: 4:1; water content: 10%; temperature: 30 °C; agitation rate: 600 rpm).

Table 1
Comparison of the transesterification conditions and performance with HMP-immobilized lipase and with other lipase systems reported in the literature.

Lipase type	Enzyme loading (U/g oil)	Optimal methanol-to-oil ratio	Water content (%)	Methanol addition strategy	FAME production rate (g/L/h)	Cost (US$/kU)	Reference
HMP-immobilized lipase	220	4:1	10	One step	42.1	1.07	This study
Novozyme 435	400	1:1	0	Stepwise	41.2	3.2[b]	[26]
Lipase AK	330	4.5:1	0	Stepwise	3.48	0.17[a]	[36]
Novozyme 435	1000	3:1	2.53	Stepwise	34.9	3.2[b]	[37]
Chirazyme L-2	44.3	3:1	0	Stepwise	0.87	NA	[38]
Candida sp. 99–125	3000	3:1	0	Stepwise	16.67	NA	[39]
Rhizopus oryzae + Chirazyme L-2	54	4.5:1	10	Stepwise	36.79	NA	[40]
Rhizopus oryzae supported by polyurethane foam	NA	3:1	12.6	Stepwise	8.99	NA	[41]

FAME: fatty acid methyl ester.
NA: not available.
[a] Calculated by the market price of lipase AK.
[b] Calculated by the market price of Novozyme 435.

This suggests that HMP-lipase seems to be suitable for industrial applications.

3.3. Effects of environmental factors on HMP-lipase catalyzed transesterification

Literature shows that the performance of lipase-catalyzed transesterification is mainly affected by temperature, agitation rate, water content, and methanol/oil ratio [27–32]. Therefore, effects of the forgoing four factors on transesterification catalyzed by HMP-lipase were investigated. As shown in Fig. 3a, the maximum initial rate occurred at 40 °C and decreased as the temperature was higher or lower than 40 °C. However, although the rate at 40 °C was slightly higher than that at room temperature (RT, about 30 °C), the energy consumption at 40 °C was markedly higher than at RT. Hence, considering both performance and energy consumption, the following experiments in this study were all performed at RT.

Fig. 3b shows the influence of agitation rate on transesterification. The initial transesterification rate did not vary significantly when the agitation rate was increased from 200 to 650 rpm, indicating that the agitation rate, over the range of 200–650 rpm, was not an important factor affecting the transesterification rate. To lower the energy consumption, an agitation rate of 200 rpm is considered as the preferable condition.

3.4. Effects of water contents and methanol loading on HMP-lipase catalyzed transesterification

The amount of water associated with the enzyme is usually a key factor in the process of lipase-catalyzed transesterification [33]. Thus, the effect of water content on transesterification was also examined in this study. As indicated in Fig. 4a, when the water content was less than 10%, the transesterification rate tended to increase with increasing water content. However, the transesterification rate decreased when the water content exceeded 10%, suggesting that a water content of 10% was sufficient for the HMP-lipase to carry out the transesterification reactions. The reason why higher water contents resulted in a lower reaction rate might be because hydrolysis of oil to fatty acids was activated when the water is abundant [34] due to the increase in the available interfacial area. As lipase usually catalyzes hydrolysis of oil in the aqueous solution, the increase in interfacial area would stimulate the competing hydrolysis reaction, thereby retarding the transesterification reactions [34].

Moreover, the ratio of the two reactants of transesterification, namely methanol and oil, is also recognized as a crucial factor that needs to be optimized. The effect of methanol-to-oil molar ratio on

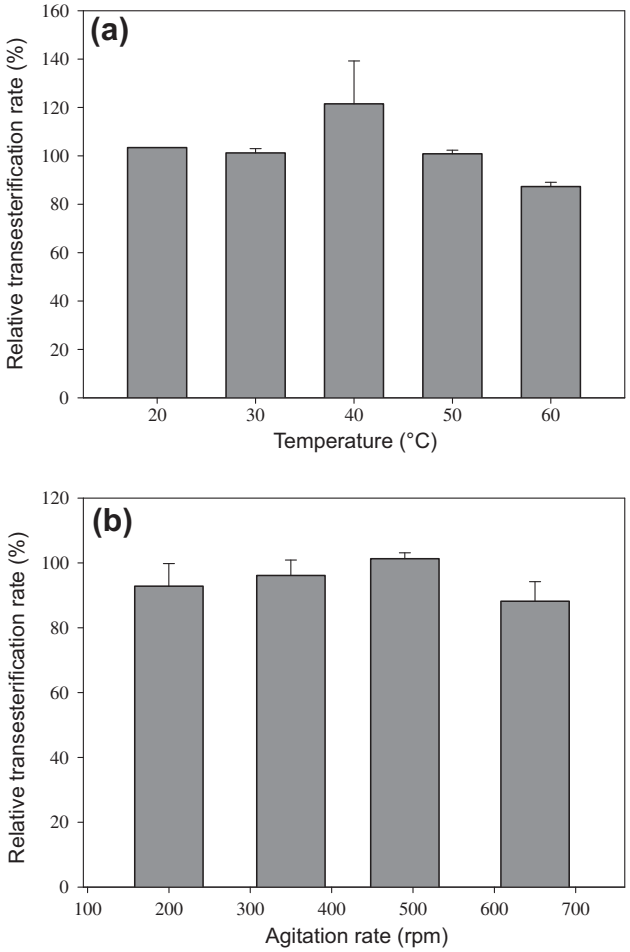

Fig. 3. Effects of (a) temperature (agitation rate at 600 rpm) and (b) agitation rate (temperature: 30 °C) on transesterification activity of the HMP-immobilized lipase (immobilized lipase loading 300 U/mL; methanol-to-oil molar ratio: 4:1; water content: 10%).

HMP-lipase-catalyzed transesterification was investigated. As shown in Fig. 4b, the maximum transesterification rate (43.5 g/l/h) occurred for the methanol concentration range of 6–13% (representing a methanol-to-oil molar ratio of 1.8 to 4.1). This maximum transesterification rate is higher than that obtained from using other types of lipases, including commercial enzymes such as Novozyme 435 [4] (Table 1). Moreover, the high optimal methanol-to-oil ratio of 4.1 implies that HMP-lipase can tolerate a high

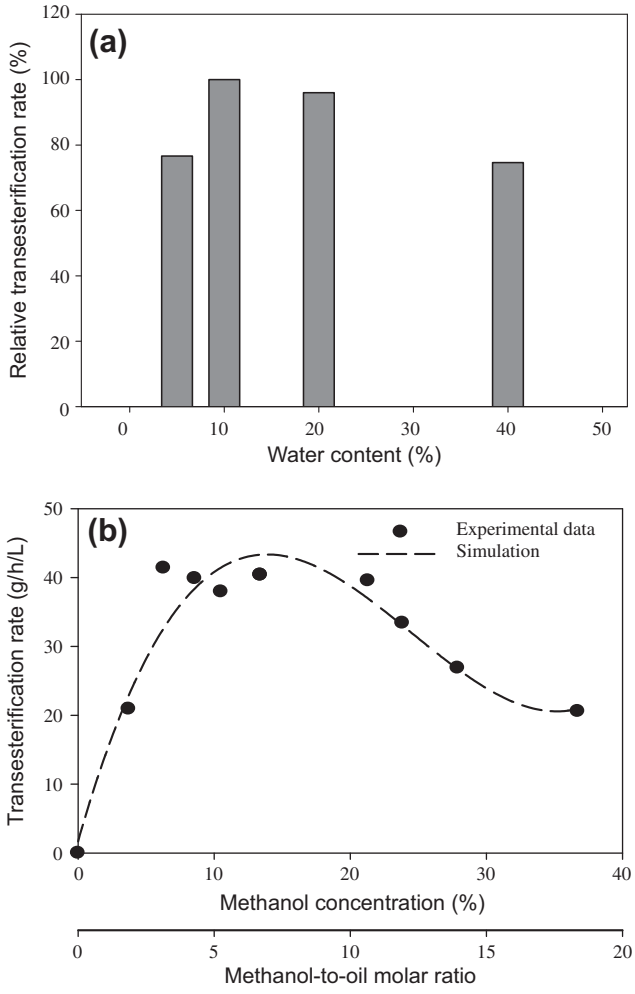

Fig. 4. Effects of (a) water content (methanol-to-oil molar ratio at 4/1) and (b) methanol-to-oil molar ratio (water content at 10%) on transesterification activity of the HMP-immobilized lipase (immobilized lipase loading 300 U/mL; temperature: 30 °C; agitation rate: 600 rpm).

concentration of methanol, which is known to repress the transesterification activity when free lipase is used [12]. Table 1 shows the comparison of the transesterification performance of using HMP-lipase and of using other lipase systems in related studies. It shows that the proposed HMP-immobilized lipase has advantages of higher methanol and water tolerance with a comparable or higher transesterification activity when compared with other lipase systems. Hence, the HMP-lipase seems to be a promising biocatalyst for enzymatic synthesis of biodiesel.

3.5. Effects of methanol addition strategy on HMP-lipase catalyzed transesterification

The transesterification activity of most lipases could be inhibited by an excessive amount of methanol [12]. An effective way to avoid methanol repression is through stepwise addition of methanol during the course of transesterification [35]. In this work, transesterification by one-step addition (whole dosing) or stepwise addition of methanol with the molar ratio of 4:1 was performed (Fig. 5). Using the one-step addition strategy, the conversion of oil to FAME reached ca. 70% in 12 h with a production rate of 39.5 g/L/h. In contrast, while the time needed to achieve the same conversion by stepwise addition was much longer (28 h) than the one-step addition approach. This demonstrates that HMP-lipase

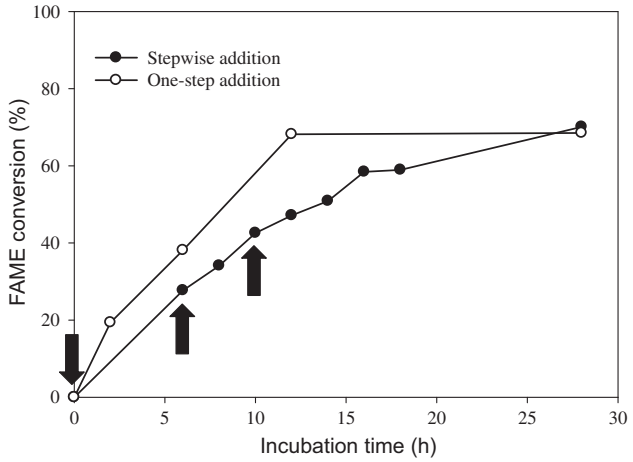

Fig. 5. Conversion of fatty acid methyl ester (FAME) by one-step and stepwise addition of methanol. The arrows indicate the addition of methanol (immobilized lipase loading 300 U/mL; methanol-to-oil molar ratio: 4:1; water content: 10%; temperature: 30 °C; agitation rate: 600 rpm).

could indeed tolerate high methanol concentration in transesterification, and thus the stepwise feeding strategy is no longer needed. In addition, due to a higher initial methanol concentration, using one-step addition significantly shortened the time required to achieve the maximum conversion. This is another way to reduce the operation costs for the enzymatic biodiesel synthesis.

4. Conclusions

Burkholderia lipase was successfully immobilized onto self-synthesized hydrophobic magnetic particles (HMPs). The immobilized lipase could be repeatedly used six times for transesterification without loss of its activity. The optimal conditions for transesterification by the developed HMP-immobilized lipase were room temperature, 200 rpm, 10% water content, and a methanol to oil molar ratio of 4:1. Under these conditions, the conversion of oil to fatty acid methyl esters was about 70% and the biodiesel a production rate was 43.5 g/L/h.

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