Journal of Molecular Catalysis B: Enzymatic 67 (2010) 52–59

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Mixed lipases for efficient enzymatic synthesis of biodiesel from used palm oil and ethanol in a solvent-free system

Ketsara Tongboriboon, Benjamas Cheirsilp*, Aran H-Kittikun

Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, 15 Kanchanawanit Rd., Hat-Yai, Songkhla 90112, Thailand

ARTICLE INFO

Article history:
Received 12 February 2010
Received in revised form 28 June 2010
Accepted 15 July 2010
Available online 22 July 2010

Keywords:
Biodiesel
Fatty acid ethyl ester
Lipase
Transesterification
Used palm oil

ABSTRACT

The enzymatic synthesis of biodiesel from used palm oil and ethanol using immobilized lipases in a solvent-free system was attempted. Five immobilized lipases, Lipase AK from *Pseudomonas fluorescens*, Lipase PS from *Pseudomonas cepacia*, Lipase AY from *Candida rugosa*, Lipozyme TL IM from *Thermomyces lanuginosa* and Novozym 435 from *Candida antarctica*, were screened based on their catalytic activities on reactions involved in biodiesel synthesis. The combined use of Lipase AY and Lipase AK gave a higher yield of biodiesel than using Lipase AK alone. The optimal conditions for biodiesel synthesis using mixed lipases in a batch system were: 2% water content, 10% enzyme dosage and 3:1 molar ratio of ethanol to oil. The mixed lipases could be used in 15 replicates with retained relative activity higher than 50%. In a continuous system using mixed lipases packed in packed-bed reactor, >67% of biodiesel was achieved.

1. Introduction

Biodiesel is renewable energy from transesterification of fat and oil with a short chain alcohol. A chemical catalysts process is the most available technique in biodiesel plants in many countries. However, one of major economic factors in this technique is the purification of the product. Glycerol as a secondary product and large amounts of inorganic material are difficult to remove [1]. Moreover, it has several drawbacks such as: being energy intensive; the alkaline catalyst must be removed from the product; alkaline wastewater requires treatment; and free fatty acid and water interfere with the reaction [2]. Many techniques such as pyrolysis, heterogeneous solid catalyst or supercritical fluid also improve the production but it seemingly need more process development [3]. In contrast, lipase catalyst reaction is a biological reaction, and is a green technology with shorter purification steps and easier to perform [4]. The main cost of this process is the price of enzyme. However, with immobilization technique the enzyme can be reused and improved stability is also gained.

Since used palm oil is generated in large quantities after deep-frying process in a seafood processing plant in Thailand. It has therefore been considered as a cheap and renewable source for converting into biodiesel. The production of ethyl esters rather than methyl esters is also of considerable interest, because the extra carbon atom brought by the ethanol molecule slightly increases the heat content and the cetane number. Another important advantage in the use of ethanol is that the ethyl esters have cloud and pour points that are lower than the methyl esters. This fact improves the cold start [5]. Also, ethanol, as extraction solvent, is preferable to methanol because of its much higher dissolving power for oils. This could thus enable the biodiesel production in a solvent-free system. Furthermore, the transesterification of oil using bioethanol from biomass is environmental friendly [6].

Although, the addition of organic solvents could improve the mutual solubility of the substrates, the removal of organic solvent could be difficult, increase costs and be time consuming [7]. Lipase-catalyzed biodiesel production in a solvent-free medium is an alternative choice for industrial application. This is because the system presents an enormous advantage by avoiding the problems of separation, toxicity, and flammability of organic solvents. This thus lowers the cost of the final product by permitting recovery of product without a step for further organic solvent evaporation step [8].

Transesterifications of various plant oils using immobilized lipases in solvent-free system have been studied. Dossat et al. [9] and Soumanou and Bornscheuer [10] synthesized methyl esters 60% and 80%, respectively, from sunflower oil in solvent-free system using immobilized *Rhizomucor miehei*. Du et al. [11] synthesized methyl esters from soybean oil in a solvent-free system using immobilized *Candida antarctica* lipase B with a conversion

* Corresponding author. Tel.: +66 74 28 6374; fax: +66 74 44 6727.
E-mail address: benjamas.che@psu.ac.th (B. Cheirsilp).

1381-1177/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2010.07.005

higher than 80% by stepwise addition of methanol. Noureddini et al. [12] synthesized 67% of methyl esters and 65% of ethyl esters from soybean oil using immobilized *Pseudomonas cepacia* lipase.

Several researchers have reported that *C. antarctica* lipase gave the highest yield of esters more than 95% [13,14]. However, the drawback of lipase-catalyzed biodiesel production is the high price of the enzyme. Since the price of *C. antarctica* lipase is relatively expensive and its use far from practicable, the study of low-cost lipases for biodiesel production is preferable. Kaieda et al. [15] reported that lipases from a number of microorganisms are able to catalyze methanolysis with appropriate water and methanol content in the reaction mixture.

Lipases from different sources show different substrate specificity. Specificity of lipases used for biodiesel synthesis refers to their regiospecificity and specificity with respect to the length of the hydrocarbon chain of fatty acid. Most of lipases are classified into two groups: *sn*-1,3-specific, which hydrolyze ester bonds at *sn*-1 and *sn*-3 positions; and non-regiospecific or random, which act on all three positions [16]. Lipases with narrow regiospecificity are believed to be inapplicable to biodiesel production. However, some *sn*-1,3-specific lipases, like those from *Thermomyces lanuginosa* efficiently catalyze transesterifications and their yield exceeds the maximum theoretical yield of 66% under appropriate conditions by increasing the migration of acyl residue from position 2 [17].

The biodiesel synthesis by lipase was assumed to take place in either one-step of transesterification or two consecutive hydrolysis and esterification steps [18–20]. Using the combination of two lipases that one could hydrolyze oil to free fatty acid and another one could produce ester from free fatty acid would improve the production of biodiesel. Talukder et al. [21] studied the two-step lipase catalysis for production of biodiesel using isooctane as a solvent, since the activity of lipase is negatively affected by methanol. The crude palm oil was first hydrolyzed to fatty acids (FA), which was then esterified to biodiesel. It is also necessary to consider that the used oils have properties different from those of refined and crude oils. The high temperatures of cooking processes and the water from the foods accelerate the hydrolysis of triglycerides and increase the free fatty acid, di- and mono-glycerides [5]. In this study, five immobilized lipases were tested for their specificities on catalytic reactions including hydrolysis, transesterification, and esterification reactions. Combinations of lipases were tested for biodiesel (fatty acid ethyl ester) synthesis from used palm oil and ethanol. The factors influencing biodiesel synthesis by the mixed lipases were optimized in a batch system. The continuous biodiesel synthesis in packed-bed reactor by the mixed lipases was also performed.

2. Materials and methods

2.1. Materials

Used palm oil was obtained from Sea Wealth Frozen Food Co. Ltd. (Songkhla, Thailand). Lipase AK from *Pseudomonas fluorescens*, Lipase PS from *P. cepacia*, and Lipase AY from *Candida rugosa*, were obtained from Amano (Nagoya, Japan). Lipozyme TL IM from *T. lanuginosa* and Novozym 435 from *C. antarctica* were supplied by Novozyme (Bagsvaerd, Denmark). Microporous polypropylene powder, Accurel EP-100 (particle size < 400 μm), was a gift from Akzo Nobel Membrana (Obernburg, Germany). All other chemicals used were of reagent or analytical grade.

2.2. Lipase immobilization

The lipase immobilization method was modified from Soumanou and Bornscheuer [10]. Before immobilization, a pretreatment of the carrier (0.5 g of Accurel EP-100) was done by adding 2 mL of absolute ethanol for a few minutes. Then, 0.5 g of lipase dissolved in 20 mL potassium phosphate buffer (pH 7, 0.1 M) was added to the wet support. The suspension was then shaken for 30 min at room temperature. The immobilized lipase was collected by filtration, washed twice with 20 mL phosphate buffer followed by distilled water (20 mL), and dried overnight under vacuum.

2.3. Screening of lipases

The hydrolysis of used palm oil, transesterification of used palm oil with ethanol, and esterification of palmitic acid with ethanol using each immobilized lipase were investigated. In the hydrolysis reaction, a mixture of 0.168 g used palm oil and 10% water content was used as substrates. The amount of free fatty acid was determined as a product. In the transesterification reaction, a mixture of 0.168 g used palm oil and 0.029 g ethanol was used as substrates. The amount of fatty acid ethyl ester was determined as a product. In the esterification reaction, 0.168 g palmitic acid in 1 mL n-hexane was used instead of used palm oil and mixed with 0.029 g ethanol. The amount of palmitic acid ethyl ester was determined as a product. All reactions were prepared in eppendorf tubes and 10% (based on oil weight) of immobilized lipase was added as a catalyst. The reaction mixtures was incubated at 45 °C and shaken at 500 rpm in an eppendorf thermomixer (Eppendorf, Germany). For the time course studies, 5 μL of reaction medium was taken at various time intervals and was diluted in 50 μL of chloroform for analysis of the composition of the product.

2.4. Optimization of biodiesel production

The effect of mixed enzyme, water content, enzyme dosage, molar ratio of ethanol to oil on biodiesel synthesis from used palm oil and ethanol were investigated. A reaction mixture consisted of 0.168 g of used palm oil, ethanol, water and immobilized lipase were prepared in eppendorf tubes, incubated at 45 °C and shaken at 500 rpm in an eppendorf thermomixer. The reusability of immobilized lipase was tested by removing products and the reaction medium after 12 h of reaction. The new substrates were added for the next reaction. The biodiesel synthesis in the first run was defined as 100% initial activity. In continuous biodiesel synthesis, the immobilized lipase (1 g) was packed in a packed-bed column (1.0 cm i.d., 10 cm long). The substrate mixture consisted of ethanol and used palm oil was fed into the bottom of the column by peristaltic pump with a controlled flow rate at 0.25 mL/min.

2.5. Analytical methods

The chemical properties of used palm oil were determined according to the method of AOAC [22]. The components of oil phase were analyzed for triacylglycerol, diacylglycerol, monoacylglycerol, free fatty acid and fatty acid ethyl ester using a thin-layer chromatography with flame ionization detection (TLC/FID) (IATROSCAN MK5, Iatron Laboratories Inc., Tokyo) [23]. In this experiment, the percent of the peak area was assumed as the percent content of the corresponding compound. Water content in oil was determined by Karl Fischer Titrator (831 KF Coulometer, Metrohm, Switzerland). The average molecular weight of used palm oil was determined by the saponification value as 910 with an acid value of 1.24. The compositions of used palm oil were triacylglycerol 93.54%, diacylglycerol 4.82%, monoacylglycerol 1.42% and free fatty acid 0.23%. The water content in used palm oil and ethanol were 0.86% and 5%, respectively.

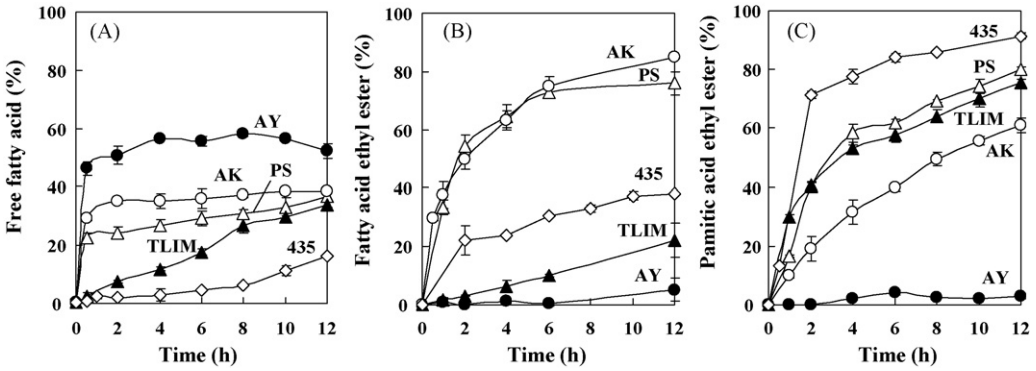

Fig. 1. Comparison of five immobilized lipases catalyzed hydrolysis (A), transesterification (B), and esterification (C) reactions. AY: Lipase AY (filled circle); AK: Lipase AK (open circle); PS: Lipase PS (open triangle); TLIM: Lipozyme TL IM (filled triangle); 435: Novozym 435 (open diamond).

3. Results and discussion

3.1. Screening of lipase

In this study, the non-specific immobilized lipases, including Lipase AK from *P. fluorescens*, Lipase PS from *P. cepacia*, Lipase AY from *C. rugosa*, Novozym 435 from *C. antarctica*, and the *sn*-1,3-specific Lipozyme TL IM from *T. lanuginosa* were screened for biodiesel synthesis from used palm oil and ethanol. The catalytic activities of five immobilized lipases on hydrolysis, transesterification, and esterification reactions were compared (Fig. 1). Fig. 1A shows the hydrolysis of used palm oil with 10% of water content using 10% of each immobilized lipase. The non-specific lipases hydrolyzed used palm oil and gave free fatty acid higher than the *sn*-1,3- specific lipase. Lipase AY was the most suitable lipase for hydrolysis reaction which could produce free fatty acid fastest and highest followed by Lipase AK and Lipase PS. The values obtained showed that the rate catalyzed by Lipase AY was 1.6 and 2 times faster than that for Lipase AK and Lipase PS, respectively.

For the transesterification of used palm oil and ethanol to biodiesel, Lipase AK and Lipase PS showed comparable high catalytic activity, while Novozym 435 and Lipozyme TL IM showed lower catalytic activity and the Lipase AY was almost inactivated (Fig. 1B). It was reported that the transesterification of several vegetable oils by bacterial lipases, including *Pseudomonas* lipase, showed a stronger preference, compared to fungal lipases such as Lipozyme TL IM [24]. Moreira et al. [2] studied the enzymatic transesterification of palm oil with ethanol using various lipases. They also found that Lipase AK gave the highest yield of ethyl ester followed by Lipase PS. In addition, they also concluded that Lipase AK is less susceptible to the inhibition effects compared with lipases from other sources such as Lipase AY and porcine pancreatic lipase in highly polar reaction media constituted with ethanol. While Kumari et al. [25] screened the lipase for transesterification of Mahua oil and ethanol and found that Lipase PS gave higher yield of biodiesel (80%) than did Lipase AK (62%). In this study, since the final yield of biodiesel produced by Lipase AK was higher than that by Lipase PS, Lipase AK was considered as the most suitable lipase

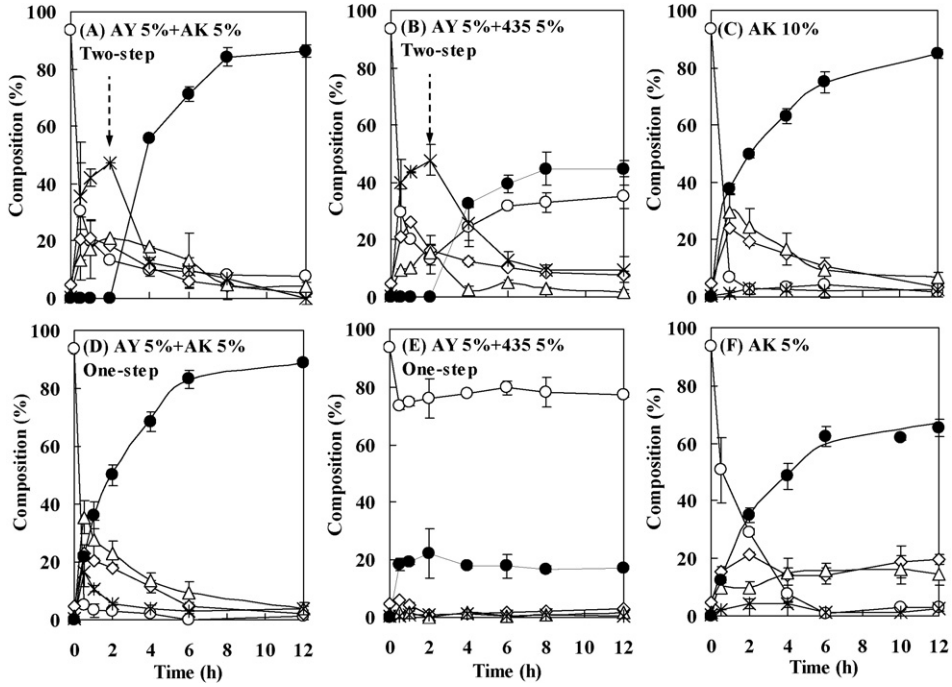

Fig. 2. Comparison of biodiesel synthesis from used palm oil and ethanol by combined lipases compared with Lipase AK alone. (A) Combined 5% Lipase AY with 5% Lipase AK in two-step reaction, (B) combined 5% Lipase AY with 5% Novozym 435 in two-step reaction, (C) 10% Lipase AK alone, (D) combined 5% Lipase AY with 5% Lipase AK in one-step reaction, (E) combined 5% Lipase AY with 5% Novozym 435 in one-step reaction, (F) 5% Lipase AK alone. Arrows separate the hydrolysis and esterification steps. Triacylglycerol (open circle); diacylglycerol (open diamond); monoacylglycerol (open triangle); free fatty acid (star); fatty acid ethyl ester (filled circle).

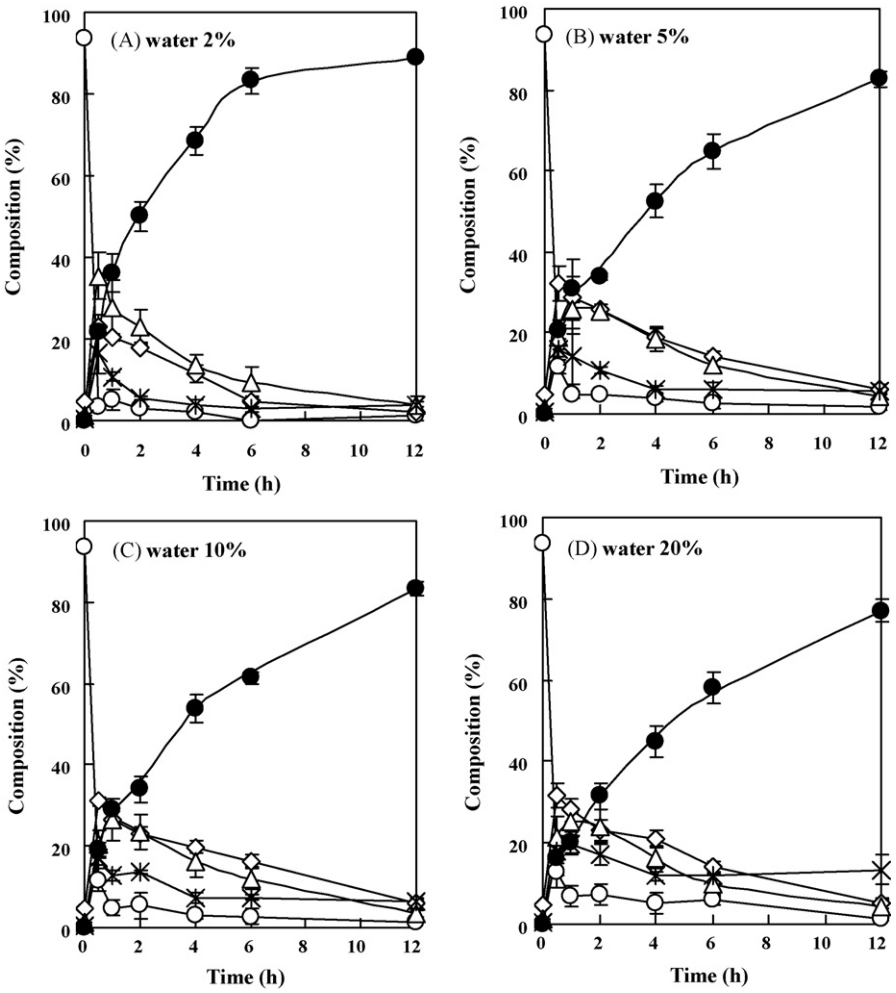

Fig. 3. Effect of water content on biodiesel synthesis. (A) 2%, (B) 5%, (C) 10% and (D) 20%. Triacylglycerol (open circle); diacylglycerol (open diamond); monoacylglycerol (open triangle); free fatty acid (star); fatty acid ethyl ester (filled circle).

for the transesterification reaction of used palm oil and ethanol to biodiesel.

In the case of the esterification reaction of palmitic acid and ethanol to palmitic acid ethyl ester, Novozym 435 gave the highest yield of palmitic acid ethyl ester followed by Lipase PS, Lipozyme TL IM and Lipase AK (Fig. 1C). The inactivation of Lipase AY was also observed in the esterification reaction. It is noted here that although Lipase AY showed high catalytic activity on the hydrolysis reaction, the catalytic activity on transesterification and esterification was very low compared to the others. This could be due the inhibitory effect of ethanol on Lipase AY. In the study of Kaieda et al. [15], they also found that *C. rugosa* lipase (Lipase AY) gave the highest catalytic activity on hydrolysis reaction. However, in the study of Shao et al. [4], they reported that Lipase AY could produce methyl esters with the conversion of 63.6% at the molar ratio of methanol to oil of 4:1, and an enzyme amount of 8% and water content of 6%.

For Novozym 435 it showed low catalytic activity on the hydrolysis and the transesterification reactions (Fig. 1A and B). However, in the esterification reaction it gave highest production rate and yield of palmitic acid ethyl ester (Fig. 1C). One possible explanation could be due to the low catalytic activity of Novozym 435 to hydrolyze an ester bond in triacylglycerol. Therefore, once free fatty acid was used it showed the high catalytic activity. In the case of Lipase AK compared with Novozym 435, Lipase AK showed higher catalytic activity on the transesterification, but lower catalytic activity on the esterification. The poor esterification efficiency of Lipase AK was also reported by other researchers [26].

3.2. Combined use of lipases

Considering the production of free fatty acid during the transesterification reaction, it is likely that successive reactions may occur (data not shown). That is, oil is first hydrolyzed to partial acylglycerols and free fatty acid, and then biodiesel are produced by the esterification of the free fatty acid and ethanol. Since Lipase AY exhibited a particularly high catalytic activity to hydrolyze used palm oil into free fatty acid (Fig. 1A), the combined use of Lipase AY with other lipases would improve the hydrolysis of triacylglycerol into free fatty acid and enhance the esterification of free fatty acid to biodiesel.

Lipase AK and Novozym 435 which showed the highest catalytic activity on the transesterification and esterification reactions, respectively, were selected for combined use with Lipase AY. The results of the combined use of Lipase AY with Lipase AK in the ratio of 1:1 (5% of each lipase) in two-step reaction, in which oil was first hydrolyzed and the free fatty acid product was esterified, and one-step reaction are shown in Fig. 2A and D, respectively. The free fatty acids were rapidly produced within 30 min by Lipase AY. Although the reaction was continued until 2 h, there was only little increase in the free fatty acid product. This could be due to either insufficient amount of water or the equilibrium of the reaction. In this

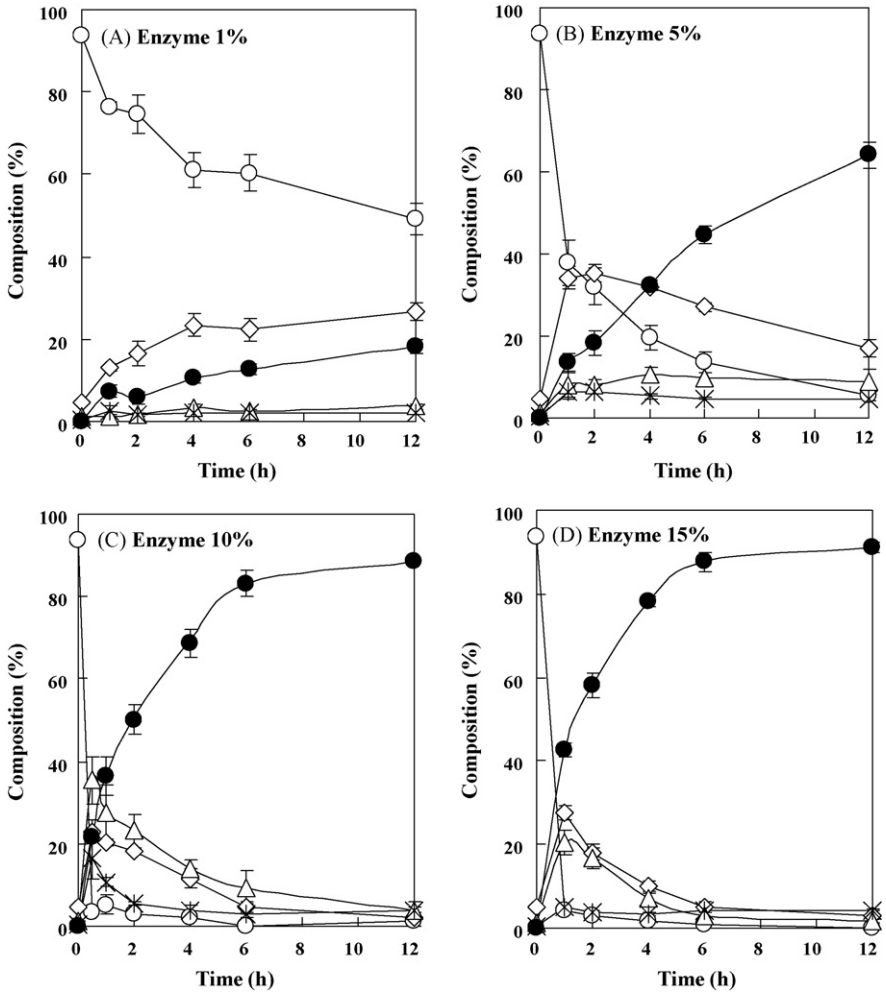

Fig. 4. Effect of enzyme dosage on biodiesel synthesis. (A) 1%, (B) 5%, (C) 10% and (D) 15%. Triacylglycerol (open circle); diacylglycerol (open diamond); monoacylglycerol (open triangle); free fatty acid (star); fatty acid ethyl ester (filled circle).

study, the one-step reaction by the combined use of Lipase AY with Lipase AK (Fig. 2D) took shorter time to reach the maximum yield of biodiesel compared with the two-step reaction (Fig. 2A). This could be due to the synergic effect of the mixed lipases which Lipase AY hydrolyzed triacylglycerol into free fatty acid and Lipase AK further converted free fatty acid and ethanol to biodiesel. Since the conversion of ethanol to biodiesel also simultaneously occurred in the mixed lipases system the inhibitory effect of ethanol on Lipase AY could be alleviated. To improve the performance of the two-step reaction, the addition of high amount of water and solvent is needed as it was reported by Talukder et al. [21]. Since we proposed the solvent-free system, the one-step reaction that could give higher yield of biodiesel without the addition of solvent was the better choice than the two-step.

The results of the combined use of Lipase AY with Novozym 435 in the ratio of 1:1 (5% of each lipase) in the two-step and one-step reactions are shown in Fig. 2B and E, respectively. In the two-step reaction, although Novozym 435 was most selective for esterification of free fatty acid, the combined use of Lipase AY with Lipase AK gave higher yield of biodiesel. Additionally, there was an increase in oil during the esterification step by Novozym 435 (Fig. 2B). These results suggested that the presence of by-product from hydrolysis step, glycerol, might promote the esterification of free fatty acid to oil instead of that to biodiesel. This phenomenon was more obvious in the one-step reaction (Fig. 2E). Therefore, in case of using Novozym 435 after the hydrolysis step not only residual water but also the by-product, glycerol, have to be removed prior to the further esterification. Contrast to the combined use of Lipase AY with Novozym 435, the combined use of Lipase AY with Lipase AK in one-step gave higher biodiesel yield in shorter time and without additional purification steps. Therefore, in this study it was concluded that the mixed Lipase AY with Lipase AK in one-step was suitable for producing biodiesel from used palm oil.

Li et al. [13] reported the transesterification of rapeseed oil by a combination of Lipozyme TI LM and Novozym 435 in a suitable ratio of 3:1. Although, Novozym 435 is an excellent lipase that is capable of efficient biodiesel synthesis at high conversion rate, the cost of it is much more expensive than the others. The use of Lipozyme TL IM reduced the necessary portion of Novozym 435 and then thus reduced the cost of the enzyme. However, the biodiesel synthesis using the cheaper lipases such as Lipase AY and Lipase AK is preferable. The improvement by the combined use of 5% Lipase AY and 5% Lipase AK in the one-step reaction (Fig. 2D) was obvious when compared with the use of Lipase AK alone at 5% (Fig. 2F). Although, this was not significantly different from the use of Lipase AK alone at 10% (Fig. 2C), the combined use of Lipase AK with Lipase AY could reduce the necessary amount of Lipase AK by half. Considering the lower cost of Lipase AY compared with the cost of Lipase AK, the combined use with Lipase AY thus makes the synthesis of biodiesel more cost-effective. In this study, the ratio of Lipase AK was further reduced to 3:1 (7.5% of Lipase AY and 2.5% of Lipase AK). However, the yield of biodiesel was reduced to 67% at

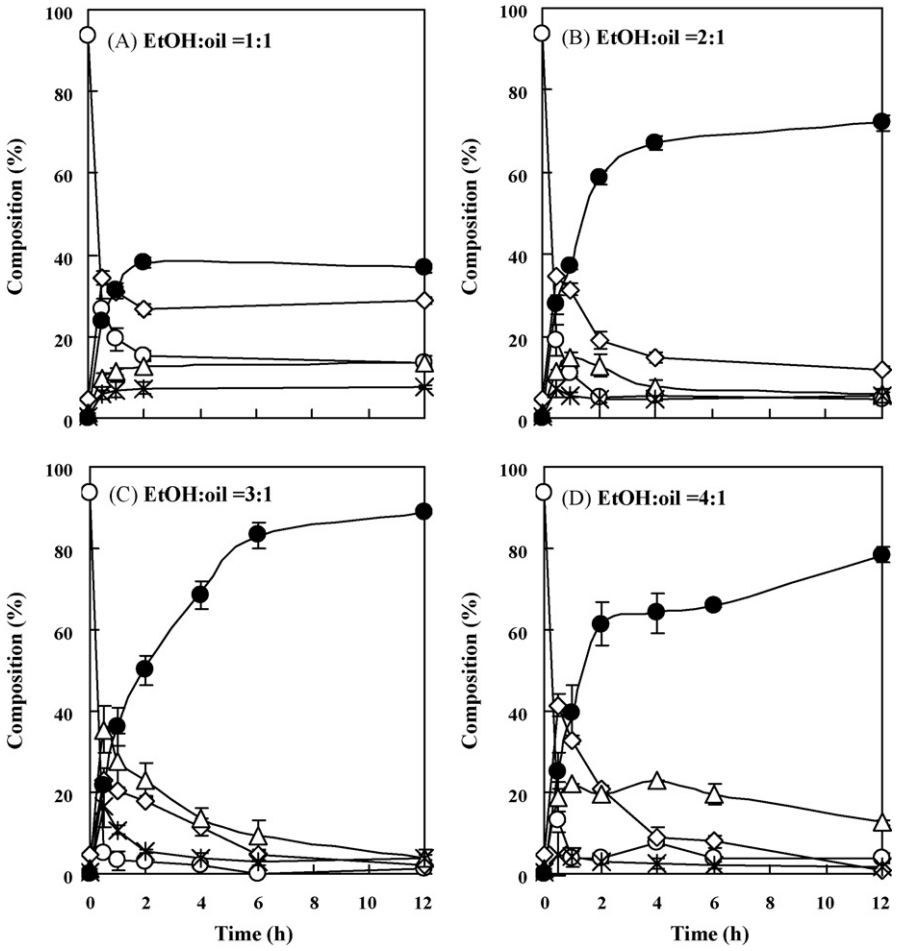

Fig. 5. Effect of molar ratio of ethanol to oil on biodiesel synthesis. (A) 1:1, (B) 2:1, (C) 3:1 and (D) 4:1. Triacylglycerol (open circle); diacylglycerol (open diamond); monoacylglycerol (open triangle); free fatty acid (star); fatty acid ethyl ester (filled circle).

12 h. Therefore, the amount of each Lipase AY and Lipase AK of 5% was chosen for further optimization of the conditions for biodiesel synthesis.

3.3. Biodiesel production by mixed lipases in a batch system

3.3.1. Effect of water content

In solvent-free system water is partitioned at a different extent among the substrates, the enzyme and the support. Lipase activity generally depends on the available interfacial area. The addition of water increases the amount of water available for oil to form an oil–water interfacial area. However, excess water may also stimulate the competing hydrolysis reaction. In practice it is more convenient to express the water content in percentage rather than in water activity because to work on a large scale the control of water activity is slightly more complicated in design and may not be convenient. Therefore, the effect of water was optimized by adding different amounts of water into the reaction media. The control was the reaction mixture without water addition which contained 2% of water content.

Generally, the transesterification of triacylglycerol yields partial acylglycerols including di- and mono-acylglycerols, free fatty acid and a final product of ester. The time courses of each composition are shown in Fig. 3. It was found that an increment in the water content from 2% to 20% increased concentration of free fatty acids and hence decreased the synthesis of biodiesel. This could be because the catalytic activity of lipases, especially Lipase AY, on hydrolysis reaction was increased when the water content increased. The equilibrium was then shifted to the production of free fatty acid. Kaieda et al. [15] reported the effect of water on the transesterification reaction by Lipase AK and Lipase PS. In their study, the optimal water content for Lipase PS was 2% while that for Lipase AK was 4%. The water content higher than the optimal level resulted in lower ester production. In this study, mixed lipases of Lipase AY and Lipase AK required lower water content of 2%. Li et al. [13] also found that the transesterification of rapeseed oils by Novozym 435

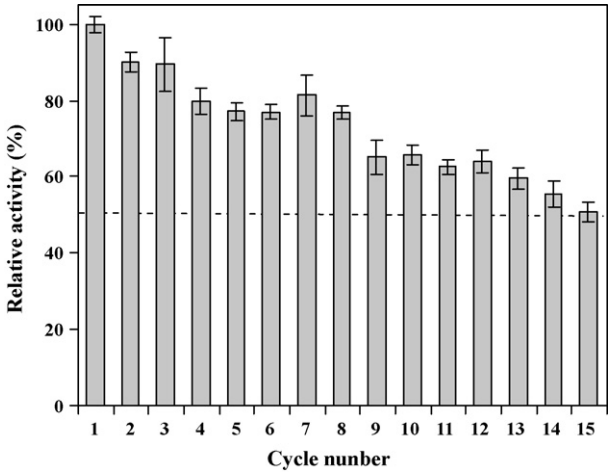

Fig. 6. Reusability of mixed lipases for biodiesel synthesis.

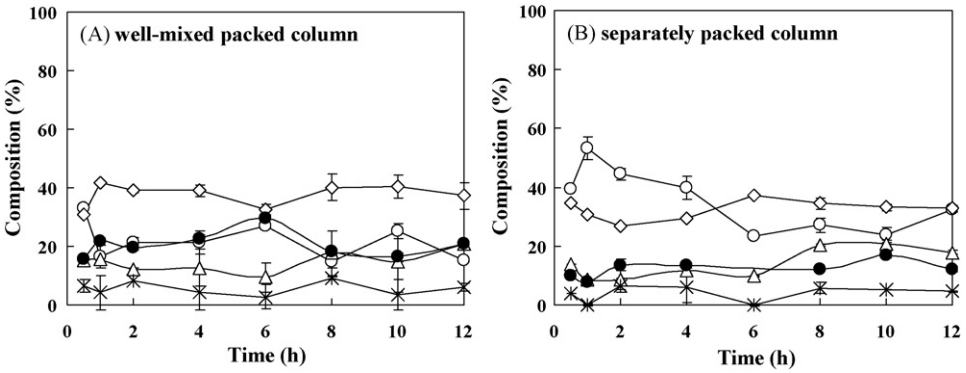

Fig. 7. Time courses of continuous biodiesel synthesis by mixed lipases. (A) Well-mixed packed column and (B) separately packed column. The substrate flow rate was 0.25 mL/min. Triacylglycerol (open circle); diacylglycerol (open diamond); monoacylglycerol (open triangle); free fatty acid (star); fatty acid ethyl ester (filled circle).

and Lipozyme TL IM decreased when increased water higher than 2%.

3.3.2. Effect of enzyme dosage

As the cost of lipase contributes significantly to the total cost of biodiesel production, the enzyme dosage should be minimized as much as possible. The effect of enzyme dosage on the synthesis of biodiesel from used palm oil and ethanol is shown in Fig. 4. The slopes of the initial part of the curves of biodiesel are proportional to the catalytic activity of the mixed lipases. The yield and initial production rate of biodiesel increased with increasing enzyme dosage. However, the biodiesel yield at the highest dosage of 15% (Fig. 4D) was similar to that of 10% (Fig. 4C). Several researchers also found that increasing of lipase dosage can improve initial production rate and yield in limited amounts because if too much enzyme is present in the reaction, especially immobilized enzyme, this could decrease the product yield. This was presumably due to the increase in the viscosity which reduced the reaction rate to the extent that an additional amount of enzyme did not help [27–29]. To minimize the cost of biodiesel synthesis an enzyme dosage of 10% was chosen for further study.

3.3.3. Effect of molar ratio of ethanol

Experiments were performed to optimize the synthesis of biodiesel by varying the molar ratio of ethanol. Optimum ethanol requirements are determined as shown in Fig. 5. The amount of ethanol added was varied from 1 to 4 molar equivalents to used palm oil. An increase in the molar ratio of ethanol from 1 to 2 resulted in an increase in the initial production rate and yield of biodiesel. The yield of biodiesel at 1 and 2 molar ratio reached stoichiometric ratio of 33% and 67%, respectively. The further increase of ethanol to 3 molar ratio increased the yield of biodiesel up to 89%, however, this was lower than the stoichiometric ratio (100%). The yield of biodiesel at 4 molar ratio was lower than that at 3 molar ratio. This could be due to the inhibitory effect of high amount of ethanol on the activity of the enzyme. It was reported that lipases can catalyze the reactions as well as the high solubility of substrates emulsion. Fatty alcohols, in which the carbon lengths are more than 3, completely dissolve in the oil in a stoichiometric amount, but the solubility of methanol and ethanol was only 1/2 and 2/3 of the stoichiometric amount, respectively [30]. Therefore, lipases could be deactivated by insoluble ethanol, which exists as drops in the oil. Soumanou and Bornscheuer [10] reported that some *Pseudomonas* strains developed substantial methanol resistance and which may be tolerant to ethanol also. Although, the mixed lipases in this study gave especially high conversion with three molar equivalents of ethanol, the stepwise addition of ethanol was also attempted. It was observed that the difference of biodiesel of which the whole of the ethanol was added in a single step (89%) and the equal amounts were added in three steps (91%) was only 2%. In this respect, the single step is a good choice since stepwise addition of the substrate does complicate the process design. In further study, the ethanol of the stoichiometric ratio of 3:1 was used to ensure higher reaction rates and minimize the diffusion limitations as ethanol could perform the solvent properties well.

3.3.4. Reusability of mixed lipases

The mixed lipases were reused in biodiesel synthesis from used palm oil and ethanol under optimum condition as described above. The reaction continued for 12 h in each batch. The relative conversion to biodiesel was determined. Fig. 6 shows the relative activity in each cycle. The activity of mixed lipases remained 90% after 3

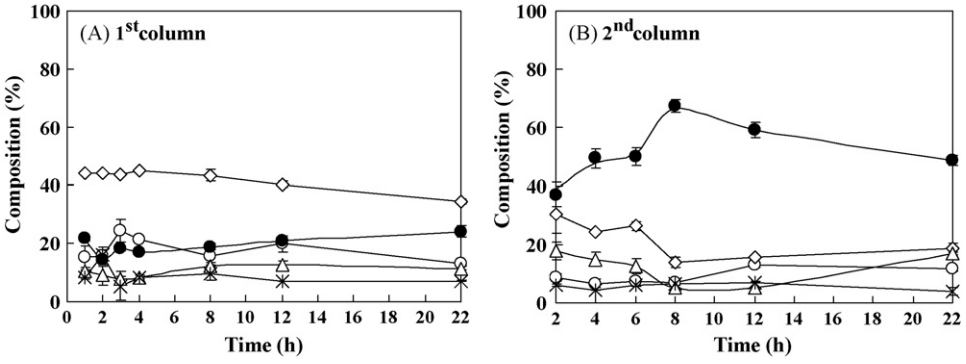

Fig. 8. Time courses of continuous biodiesel synthesis by mixed lipases in dual well-mixed packed-bed column. (A) The reaction in the first column. (B) The reaction in the second column. The substrate flow rate was 0.25 mL/min. Triacylglycerol (open circle); diacylglycerol (open diamond); monoacylglycerol (open triangle); free fatty acid (star); fatty acid ethyl ester (filled circle).

cycles and decreased to 80% after 7 cycles. However, the activity of the mixed lipases remained higher than 50% after 15 cycles. A decrease of biodiesel production during the reusing of the enzymes may have been caused by glycerol accumulation to the enzyme carrier. Li et al. [13] reported that in enzymatic biodiesel production with solvent-free or hydrophobic reaction medium, much glycerol has been found to be adsorbed on to the surface of the immobilized lipase which led to the quite short operational life of the lipases. Although, the use of the solvent was proposed in their study, the solvent-free system is more advantageous considering the cost of solvent removal after the reaction.

3.4. Biodiesel production in packed-bed reactor

Continuous biodiesel synthesis using mixed lipases was performed on a packed-bed reactor. Compared with reaction in batches the packed-bed reactor enhanced repeated use of lipases by protecting enzymes from the physical damage that an excess amount of alcohol caused. In this study, two lipases were packed in the column in two patterns. One was a well-mixed pack and another was a separate pack with Lipase AY in the bottom and Lipase AK in the top of the column. A substrate mixture of used palm oil and 3 molar equivalent of ethanol were fed into the bottom of the column at a constant flow rate of 0.25 mL/min. The temperature was controlled at 45 °C by circulating water. The results are shown in Fig. 7. The average conversion of used palm oil to biodiesel in the separately packed column was only 14% and the average residual triacylglycerol was 35% (Fig. 7B). This could be due to the inhibitory effect of ethanol on the activity of Lipase AY, because when Lipase AY was separately packed in the bottom of the column, it was faced with a high concentration of ethanol. While the well-mixed packed column showed higher average biodiesel production of 22% and the average residual triacylglycerol was 17% (Fig. 7A).

To further increase the conversion of used palm oil to biodiesel, the retention time should be increased either by decreasing the substrate flow rate or increasing the length of the column. However, since the viscosity of the substrate in solvent-free system was high, decreasing the substrate flow rate caused the separation of the oil and ethanol layer. Therefore, the length of the column was increased by increasing the number of the column. The results of biodiesel synthesis in dual well-mixed packed-bed columns are shown in Fig. 8. The biodiesel content from the second column reached maximum value of 67% at 8 h, but it decreased to 59% and 49% after 12 h and 22 h, respectively. This could be due to the irreversible inactivation of the lipases. This discrepancy may be explained by the effect of by-product of ethanolysis, glycerol. In the flow reaction, the glycerol might remain in the bottom of the column because of its high viscosity. The glycerol could disturb the diffusion of substrates to lipase molecule, and the decrease in reaction efficiency gradually increases the unreacted alcohol [31]. In repeated batch system, several methods for glycerol removal were attempted such as dialysis [32], and absorption on silica gel [27]. In continuous system, the use of solvent in which glycerol was well dissolved and therefore it did not form films on the carrier, was proposed [13].

4. Conclusions

In this study, the used palm oil was found to be an effective raw material for the enzymatic synthesis of biodiesel fuel using cheap lipases in a solvent-free system. The highest biodiesel yield could be achieved using the combination of Lipase AK and Lipase AY. The optimum conditions for biodiesel synthesis using mixed lipases were determined. The mixed lipases could be repeatedly used under the optimal conditions for 15 times with a relative activity higher than 50%. The mixed lipases also showed the possibility for their use in continuous biodiesel synthesis without the addition of solvent.

Acknowledgements

The authors are very grateful to the financial support by Prince of Songkla University and Thai Government in the fiscal year of 2009 under Grant AGR520011S. We would like to thank Amano Pharmaceuticals Inc. (Nagoya, Japan) and Novo (Denmark) for the gifts of lipase and Sea Well Frozen Food Co. Ltd., Thailand, for the gift of used palm oil. The authors thank Professor Anthony G. Hopkin for proof reading the manuscript.

References

[1] M. Mitttelbach, J. Am. Oil Chem. Soc. 67 (3) (1990) 168–170.
[2] A.B.R. Moreira, V.H. Perez, G.M. Zanin, H.F. de Castro, Energy Fuels 21 (2007) 3689–3694.
[3] Z. Helwani, M.R. Othman, N. Aziz, W.J.N. Fernando, J. Kim, Fuel Process. Technol. 90 (2009) 1502–1514.
[4] P. Shao, X. Meng, J. He, P. Sun, Food Bioprod. Process. 86 (2008) 283–289.
[5] J.M. Encinar, J.F. González, A. Rodríguez-Reinares, Fuel Process. Technol. 88 (2007) 513–522.
[6] M. Balat, H. Balat, C. Oz, Prog. Energy Combust. Sci. 34 (2008) 551–573.
[7] M. Szczesna-Antczak, A. Kubiak, T. Antczak, S. Bielecki, Renew. Energy 34 (2009) 1185–1194.
[8] B. Selmi, D. Thomas, J. Am. Oil Chem. Soc. 75 (6) (1998) 691–695.
[9] V. Dossat, D. Combes, A. Marty, Enzyme Microb. Technol. 30 (2002) 90–94.
[10] M.M. Soumanou, U.T. Bornscheuer, Enzyme Microb. Technol. 33 (2003) 97–103.
[11] W. Du, Y. Xu, D. Liu, J. Zeng, J. Mol. Catal. B: Enzym. 30 (2004) 125–129.
[12] H. Noureddini, X. Gao, R.S. Philkana, Bioresour. Technol. 96 (2005) 769–777.
[13] L. Li, W. Du, D. Liu, L. Wang, Z. Li, J. Mol. Catal. B: Enzym. 43 (2006) 58–62.
[14] Md.M.R. Talukder, S.M. Puah, J.C. Wu, C.J. Won, Y. Chow, Biocatal. Biotransform. 24 (4) (2006) 257–262.
[15] M. Kaieda, T. Samukawa, A. Kondo, H. Fukuda, J. Biosci. Bioeng. 91 (1) (2001) 12–15.
[16] K. Stransky, M. Zarevucka, Z. Kejık, Z. Wimmer, M. Mackova, K. Demnerov, Biochem. Eng. J. 34 (2007) 209–216.
[17] W. Du, Y.Y. Xu, D.H. Liu, Z.B. Li, J. Mol. Catal. B: Enzym. 37 (2005) 68–71.
[18] S. Al-Zuhair, Biotechnol. Prog. 21 (5) (2005) 1442–1448.
[19] S. Al-Zuhair, F.W. Ling, L.S. Jun, Process Biochem. 42 (2007) 951–960.
[20] B. Cheirsilp, A. H-Kittikun, S. Limkatanyu, Biochem. Eng. J. 42 (2008) 261–269.
[21] Md.M.R. Talukder, J.C. Wu, N.M. Fen, Y.L.S. Melissa, Biochem. Eng. J. 49 (2010) 207–212.
[22] A.O.A.C., Official Method of Analysis of the Association of Official Chemists, 15th ed., The Association of official Analysis Chemists Inc., Virginia, 1990.
[23] W. Kaewthong, A. H-Kittikun, Enzyme Microb. Technol. 35 (2004) 218–222.
[24] H.M. Ghazali, S. Hamidah, Y.B. Che Man, J. Am. Oil Chem. Soc. 72 (6) (1995) 633–639.
[25] V. Kumari, S. Shah, M.N. Gupta, Energy Fuels 21 (2007) 368–372.
[26] M.L. Foresti, M. Pedernera, V. Bucala, M.L. Ferreira, Enzyme Microb. Technol. 41 (2007) 62–70.
[27] Y. Yesiloglu, J. Am. Oil Chem. Soc. 81 (2) (2004) 157–160.
[28] A.F. Hsu, K.C. Jones, T.A. Foglia, W.N. Marmer, Biotechnol. Lett. 25 (2003) 1713–1716.
[29] S. Shah, S. Sharma, M.N. Gupta, Energy Fuels 18 (2004) 154–159.
[30] Y. Shimada, Y. Watanabe, A. Sugihara, Y. Tominaga, J. Mol. Catal. B: Enzym. 7 (2002) 133–142.
[31] Y. Watanabe, Y. Shimada, A. Sugihara, H. Noda, H. Fukuda, Y. Tominaga, J. Am. Oil Chem. Soc. 77 (4) (2000) 355–360.
[32] K.B. Bako, F.C.S. Kova, L. Gubicza, J.K. Hansco, Biocatal. Biotransform. 20 (2002) 437–439.