Journal of Molecular Catalysis B: Enzymatic 73 (2011) 74–79

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

Biocatalytic esterification of palm oil fatty acids for biodiesel production using glycine-based cross-linked protein coated microcrystalline lipase

Marisa Raita [a], Thanaporn Laothanachareon [b], Verawat Champreda [b,*], Navadol Laosiripojana [a]

[a] Joint Graduate School for Energy and Environment (JGSEE), King Mongkut's University of Technology Thonburi, Bangmod, Bangkok 10140, Thailand
[b] Enzyme Technology Laboratory, National Center for Genetic Engineering and Biotechnology, 113 Thailand Science Park, Paholyothin Road, Klong Luang, Pathumthani 12120, Thailand

ARTICLE INFO

Article history:
Received 20 January 2011
Received in revised form 29 July 2011
Accepted 30 July 2011
Available online 17 August 2011

Keywords:
Biodiesel
Crosslinked protein coated microcrystals
Esterification
Lipase
Palm fatty acid distillate

ABSTRACT

Conversion of feedstocks containing high free fatty acid contents to alkyl esters is limited by the currently used alkali-catalyzed biodiesel synthesis process. In this study, esterification of palm fatty acids to ethyl esters was studied using heterogeneous cross-linked protein coated microcrystalline (CL-PCMC) lipase. Optimization of biocatalyst synthesis by variation of matrix components and organic solvents showed that highly active CL-PCMCs could be prepared from *Thermomyces lanuginosus* lipase with glycine as the core matrix in acetone. The optimized reaction contained 20% (w/w) glycine-based CL-PCMC-lipase, a 1:4 fatty acid molar equivalence to ethanol in the presence of an equimolar amount of *tert*-butanol which led to production of 87.2% and 81.4% (mol/mol) of ethyl ester from palmitic acid and industrial palm fatty acid distillate (PFAD), respectively after incubation at 50 °C for 6 h. CL-PCMC-lipase is more catalytically efficient than protein coated microcrystalline (PCMC) lipase, Novozyme®435 and Lipolase 100T for both free fatty acids and palm fatty acid distillate. The CL-PCMC-lipase showed high operational stability with no significant loss in product yield after 8 consecutive batch cycles. The glycine-based microcrystalline lipase is thus a promising alternative economical biocatalyst for biodiesel production from inexpensive feedstocks with high free fatty acid contents.

1. Introduction

Biodiesel is an alternative fuel for diesel engines produced mainly by transesterification of vegetable oils or animal fats with short chain alcohols. In Thailand and Southeast Asian countries, the main local feedstock for biodiesel production is purified palm oil (PPO), which is derived from refinery crude palm oil (CPO), a product of the palm oil processing industry. Typically, an alkali-catalyzed transesterification reaction is used for conversion of triacylglycerol in the feedstock to fatty acid alkyl esters. However, this process is sensitive to free fatty acids (FFAs), which cause undesirable saponification, leading to low product yields and complication in the subsequent separation steps [1]. Due to increasing demand of palm oil for biodiesel and food industry, the price of crude palm oil has been increasing in the past few years (1100 US$/ton in 2011). Conversion of cheaper alternative feedstocks to biodiesel is thus of interest in order to economically compete with petroleum-based fuel. Palm fatty acid distillate (PFAD) is a by-product from the refinement of CPO to PPO which has a high free fatty acid content. Typically, 3–10% of PFAD is obtained from crude palm oil, which is produced at 800,000 tons/year in Thailand, making it an economically promising feedstock for biodiesel production. The development of an efficient process to convert PFAD and feedstocks containing high FFAs to biodiesel is thus needed for improving the economics of the biodiesel industry.

Typically, conversion of FFAs to biodiesel can be carried out by acid-catalyzed esterification processes using strong acids, mostly H_2SO_4. However, a major limitation of the homogeneous acid catalyzed process is the difficulty in catalyst recovery and waste treatment, as well as corrosion of the equipment, which thus increase the overall cost of the process. Several alternative approaches for biodiesel production from feedstocks containing high FFA content have been reported, including heterogeneous acid catalyzed processes [2] and the non-catalytic or catalytic near- and super-critical methanol processes [3,4]. However, these approaches still have drawbacks due to high cost of the heterogeneous catalysts and the high energy consumption of the thermal processes. Research on a less energy-intensive and environmentally-friendly alternative process for biodiesel synthesis from PFAD or other feedstocks containing high FFA contents is thus of great interest.

Biocatalytic processes employing lipase biocatalysts have gained increasing interest for industrial biodiesel production [5]

Abbreviations: CL-PCMCs, crosslinked protein coated microcrystals; EtOH, ethanol; FFA, fatty acid; FAEE, fatty acid ethyl ester; PFAD, palm fatty acid distillate; *t*-BuOH, *tert*-butanol.

* Corresponding author. Tel.: +66 2564 6700x3473; fax: +66 2564 6707.
E-mail address: verawat@biotec.or.th (V. Champreda).

1381-1177/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2011.07.020

which allows high conversion efficiency of feedstocks containing glycerides with high FFA contents under mild operational conditions with no requirement for subsequent wastewater treatment [6]. Development on enzymatic processes for biodiesel production has been focused on the cost reduction for lipases and improvement of the enzyme's operational time and reusability, which would benefit the commercialization of the biocatalytic process. Immobilization is a potential approach for optimizing the operational performance of enzymes and cost of biocatalysts in industrial processes, especially for non-aqueous systems. Enzyme immobilization by precipitation is a cost efficient approach for biocatalyst preparation for use in organic media. This alternative immobilization method involves several forms of biocatalysts including cross-linked enzyme aggregates (CLEAs) [7], cross-linked enzyme crystals (CLECs) [8], and protein-coated microcrystals (PCMCs) [9–12]. Recently, cross-linked PCMCs (CL-PCMCs) have been reported as an improved biocatalyst design based on conventional PCMCs [13]. CL-PCMCs are characterized as a cross-linked enzyme layer on the surface of micron-sized inner core matrix, which can be prepared by rapid dehydration and co-precipitation of enzyme and the matrix component in an organic solvent, the same as for conventional PCMC preparation, with an extra step on enzyme covalent crosslinking. CL-PCMCs possess several advantages over existing carrier-based or carrier-free immobilization methods, including a low mass-transfer limitation and high catalytic performance, with improved stability and reusability. In this study, the synthesis of CL-PCMC lipase biocatalysts has been optimized using various core matrices and precipitating organic solvents for efficient esterification of FFAs, used as model reactants and PFAD from palm oil industry. The effects of reaction parameters have been investigated based on the biocatalyst's reactivity on ethyl ester synthesis. The results of this study could be applied for synthesis of biodiesel from feedstocks with high fatty acid content, thus providing an economically and environmentally attractive approach for biodiesel production.

2. Materials and methods

2.1. Materials

Palm fatty acid distillate (PFAD) was obtained from the Pathum Vegetable Oil, Co. Ltd. (Pathumthani, Thailand). The PFAD sample contained 93% (w/w) free fatty acid (45.6% palmitic, 33.3% oleic, 7.7% linoleic as the major FFA) and the rest comprising triglycerides, diglycerides, monoglycerides and trace impurities. Free fatty acids (palmitic, oleic and linoleic acids) and fatty acid ester standards were obtained from Sigma–Aldrich. Liquid *Thermomyces lanuginosus* lipases, from genetically modified *Aspergillus* sp., DELIP 50L (50 KLU/g) was supplied by Flexo Research, Pathumthani, Thailand (1 KLU is defined as the amount of enzyme liberating 1 mmol of titratable butyric acid from tributyrin in 1 min). Lipase activity on *p*-nitrophenyl palmitate was assayed according to Raita et al. [12]. Novozymes® 435 (immobilized *Candida antarctica* lipase B) and Lipolase 100 T (granulated silica immobilized *T. lanuginosus* lipase) were from Novozymes (Bagsvaerd, Denmark). Chemicals and reagents were analytical grade and obtained from major companies. All reagents were dehydrated with 3 Å molecular sieves (Fluka, Buchs, Switzerland) before use.

2.2. Optimization of CL-PCMC-lipase preparation

CL-PCMC-lipase was prepared based on the method modified from Shah et al. [13] with modification on synthesis conditions and variation in core matrices and solvents. Commercial lipase preparation DELIP 50L (192 ml) was clarified by centrifugation (12,000 × *g*, 10 min) and pre-concentrated (3×, to 64 ml) using ultrafiltration on a Minimate tangential flow filtration system using a Minimate TFF capsule with 10 kDa MWCO membrane (Pall, Easthills, NY, USA). For optimization of CL-PCMC-lipase synthesis, 1.5 volume (96 ml) of a saturated solution of the matrix component (potassium sulphate, glucose, MOPS (3-morpholinepropanesulfonic acid), or glycine) was added to 1 volume of the concentrated lipase solution. This combined mixture was then added drop-wise to a stirring vial (150 rpm) containing 7.5 volume of organic solvent (acetone, ethanol, or *tert*-butanol). The precipitate was obtained by centrifugation at 2200 × *g* for 5 min and then washed thrice with 0.5 volume of the corresponding solvent. The enzyme precipitate (*i.e.* PCMCs) was resuspended in 160 ml of the solvent, followed by addition of 3.2 ml of glutaraldehyde (25% v/v in water). The mixture was incubated at 4 °C with stirring at 300 rpm for 1 h and then washed with the corresponding solvent. The air dried precipitate (265 mg from initial lipase solution of 1 ml) was used as the biocatalyst in this study. The protocol for preparation of the optimal glycine-based CL-PCMC-lipase is shown in Fig. 1. Protein content was determined at the PCMC stage with Bio-Rad protein assay reagent based on Bradford's method using bovine serum albumin as the standard.

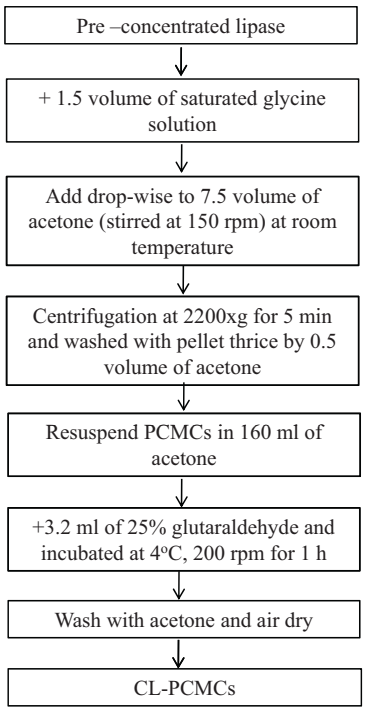

Fig. 1. Preparation of glycine-based CL-PCMC-lipase.

2.3. Lipase catalyzed esterification

For the optimized reaction, 250 mg of FFA or PFAD and ethanol was reacted in a molar ratio of 4:1 ([EtOH]/[FFA]) in the presence of *tert*-butanol at a 1:1 molar ratio ([*t*-BuOH]/[FFA]). The optimal CL-PCMC-lipase prepared with glycine and acetone was added at 20% (w/w based on FFA or PFAD) in the reaction and incubated at 50 °C on a vertical rotator. Samples were withdrawn at time intervals. The samples (2 μl) were diluted with hexane (10 μl) and mixed with lauric acid methyl ester (5 μl) as an internal standard. The amount of esters formed was then determined by gas chromatography according to Raita et al. [12]. The FAEE production yield (%) is the amount of fatty acid ethyl esters converted from available fatty acid equivalence (as FFAs and glycerides) on a molar basis. For reusability study, the biocatalyst was recovered by centrifugation,

washed with 1 ml of the organic solvent twice (if indicated), and air-dried before use in the next batch. The reactions were done in triplicate and standard deviations were reported for all experimental results.

2.4. Gas chromatography analysis of alkyl esters

The alkyl esters were analyzed by gas chromatography on a Shimadzu 2010, equipped with a flame ionization detector (Shimadzu, Kyoto, Japan) and a polyethylene glycol capillary column (Carbowax 20 M, 30 m × 0.32 mm, Agilent Technologies, Santa Clara, CA). The column oven temperature was at 200 °C, with injector and detector temperatures at 250 and 260 °C, respectively. Helium was used as the carrier gas at a constant pressure of 64.1 kPa with linear velocity at 25 cm/s. The amount of FAEE was determined based on the standard curves using the corresponding esters.

2.5. Physical analysis techniques

The structure and morphology of the CL-PCMC-lipase was analyzed by scanning electron microscope (SEM) using a JSM-6301F Scanning Electron Microscope (JEOL, Tokyo, Japan). The samples were dried and coated with gold for analysis. An electron beam energy of 5 kV was used for analysis. X-ray diffraction (XRD) data were collected at room temperature on a Rigaku TTRAX III X-ray diffractometer using Cu Kα radiation (λ = 1.5418 Å). The sample was scanned in the 2θ value of 10–45° at a rate of 2°/min.

3. Results and discussion

3.1. Optimization of CL-PCMC synthesis

In the first stage, the synthesis conditions for CL-PCMC-lipase biocatalyst were optimized based on their reactivity towards production of ethyl palmitate from palmitic acid used as a model reactant. *Thermomyces* (*Humilcola*) *lanuginosus* lipase was used for its high reactivity on biodiesel production from palm oil feedstock [12]. The 3× pre-concentrated enzyme in solution showed high hydrolysis activity towards *p*-nitrophenyl palmitate with the specific activity of 6.93×10^{-3} IU/mg equivalent to the volumetric activity of 0.27 IU/ml, and was used for preparation of CL-PCMC conjugate. Two key factors for CL-PCMC synthesis were investigated, namely (i) the matrix components, which were selected to represent inorganic and organic matrices (K_2SO_4, glucose, MOPS, and glycine) and (ii) the precipitating organic solvents (ethanol, *tert*-butanol, and acetone). The reactions were optimized based on ethanolysis due to the *T. lanuginosus* lipase's higher stability in ethanol in comparison to methanol, which showed inactivation effect to the lipase from *T. lanuginosus* [12]. The use of ethanol as nucleophile is also advantageous for the development of green biodiesel, where all the reactants are from recyclable biological sources.

The highest reactivity of lipase biocatalyst was obtained using glycine as the matrix component with acetone as the precipitating organic solvent (Table 1). The glycine-based CL-PCMCs formed fine crystalline particles and had the protein content of 155 μg/mg of CL-PCMCs. This optimized combination led to FAEE synthesis at 85.0% yield after 6 h in the presence of *tert*-butanol under the optimal synthesis conditions while the control reaction with no biocatalyst led to no detectable products under the same conditions. Lower FAEE yields were obtained with glycine-based CL-PCMCs using ethanol and *tert*-butanol as the precipitating solvents or CL-PCMCs prepared using other matrix components. K_2SO_4 is the most commonly used matrix for synthesis of several forms of biocatalyst *e.g.* PCMCs and CL-PCMCs of various enzymes [10,12,13]. However, lower FAEE yields were obtained for the K_2SO_4-based CL-PCMC-lipase prepared using different solvents (range: 69.9–74.7%). Glucose and MOPS were found to be unsuitable matrix for CL-PCMC-lipase preparation due to their lower catalytic efficiency per weight basis and the low amount of biocatalysts obtained after precipitation. Different core matrices including salts, sugars, amino acids, and inorganic/organic buffer substances with protic or aprotic solvents were previously used for optimization of PCMC synthesis from various enzymes [14,15]. Amino acids, including L-glutamine and DL-valine were previously used for preparation of PCMCs for biocatalysts and for vaccine formulation [9,16,17]. The effects of core matrix components in microcrystalline biocatalyst preparation are generally dependent on manipulation of the micro-environment of the enzyme and the physical properties and morphology of the carrier, which are the result of the intrinsic properties of the carrier, coupled with the choice of precipitating solvent [14]. Matrix components prepared from solid-state buffer substances as the core matrix have been reported to give biocatalysts with improved reactivity and stability as demonstrated for PCMCs of subtilisin prepared with organic or inorganic buffer carriers (either as a mixture of the Na^+ salt and the zwitterionic form or as a one-component solid state buffer *e.g.* Na-AMPSO, $NaCO_3$, and $NaHCO_3$) in comparison to that prepared using the non-buffered inert K_2SO_4 [14]. In PCMCs, the intimate association of the enzyme and solid-state buffer compound would allow efficient equilibration of the ionization state of the biocatalyst. To our knowledge, our study is the first report on the use of zwitterionic glycine as the core matrix for biocatalyst synthesis for application in water immiscible organic solvent systems. Addition of external glycine/Na^+ salt was previously used for ionization state control for biocatalysis in organic media [18]. However, due to the use of only the zwitterioinic form of glycine and its pK_a in aqueous system, the mechanism of improved catalytic performance of the optimized glycine-based CL-PCMC lipase may not be clearly understood based on the hypothesis on ionization state control of the enzyme by solid-state buffer. The finding thus suggested further detailed study on the roles of core matrix component and its interaction with the enzyme in CL-PCMC preparation. Acetone was used as the precipitating solvent for preparation of various forms of lipase biocatalyst *e.g.* PCMCs [10,12], CL-PCMCs [13], and acetone rinsed enzyme preparation (AREP) [19]. The preference of acetone as the precipitating solvent to a series of alcohols with different polarities was different to that reported for preparation of different biocatalyst designs *e.g. n*-propanol for PCMCs of subtilisin [9,14] and 1,2-dimethoxyethane for enzymes prepared and rinsed with organic solvent (EPROS) of lipases [20]. The result thus suggests the prerequisite to screen the best combination of the core matrix and precipitating solvent for obtaining a high performance biocatalyst for a specific process in organic media.

The physical characteristics of CL-PCMC-lipase were examined using SEM (Fig. 2). The biocatalysts had a variable size distribution in the micron size range of 10–20 μm. The overall surface of CL-PCMC-lipase was different to the glycine salt control, which showed

Table 1
Optimization of CL-PCMC synthesis. CL-PCMC-lipases were prepared with different matrix component in different organic solvents.

Matrix component	FAEE yield (%)		
	Acetone	Ethanol	*tert*-Butanol
K_2SO_4	69.9 ± 3.3	74.7 ± 3.3	73.6 ± 2.2
Glucose	67.2 ± 2.5	49.8 ± 1.2	NA
MOPS	45.9 ± 1.5	NA	NA
Glycine	85.0 ± 2.3	17.5 ± 0.2	27.0 ± 0.7

The reactions contained 250 mg of palmitic acid, 4:1 [EtOH]/[FFA] molar ratio, in the presence of 1:1 [*t*-BuOH]/[FFA] molar ratio with 20% (w/w) CL-PCMC-lipase. The reactions were incubated at 50 °C for 6 h.
NA: not analyzed due to no or low amount of CL-PCMCs obtained.

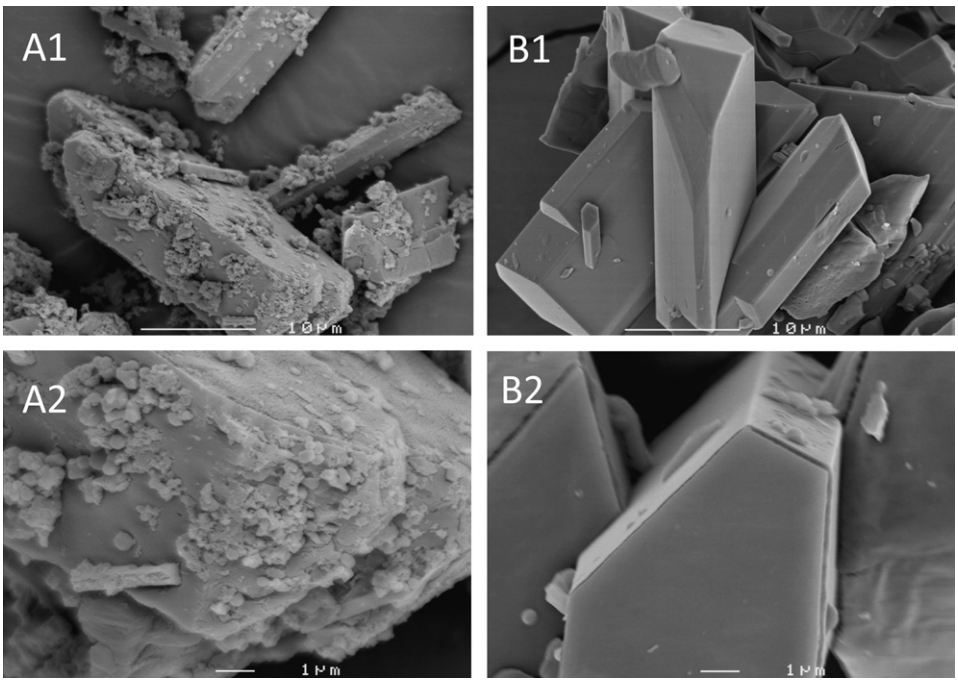

Fig. 2. SEM analysis of CL-PCMC-lipase. (A) CL-PCMC-lipase prepared from glycine in acetone; (B) glycine crystals control, no lipase added.

a homogeneous monoclinic crystal structure. The biocatalysts were formed as protein aggregates on the glycine crystal surface, suggestive of enzyme molecule aggregation on the amino acid crystals. XRD analysis showed that the glycine-based CL-PCMCs were highly crystalline (Fig. 3). Signature peaks of α-glycine were identified (JCPDS number 32-1702), reflecting crystal structure of the core matrix. The formation of enzyme layer on the salt crystals in CL-PCMCs results in higher exposed reactive surface area of the biocatalyst (based on the same enzyme content on weight basis) and lower mass transfer limitation to the lipase active site in comparison to CLEAs or CLECs [7,8]. However, characterization of the biocatalyst surface area using BET surface area analysis was limited by the low melting temperature of glycine.

3.2. Effects of reaction parameters on esterification

Initial trials on optimization of the operational conditions for biodiesel synthesis were focused on the effects of nucleophile and co-solvent ratios to free fatty acids based on esterification of palmitic acid (Fig. 4). Systematic optimization of ethanol and *tert*-butanol contents was investigated for all combinations. The reaction temperature in this study was set at 50 °C to allow complete solubilization of FFAs. In most cases, increasing the ethanol:FFAs ratio led to increased FAEE yields at all *tert*-butanol ratios. The optimal [EtOH]/[FFA] ratio of 4:1 is comparable to previous reports on biocatalytic transesterification of different vegetable oils [11,12]. The presence of *tert*-butanol at a 1:1 molar ratio ([*t*-BuOH]/[FFA]) in the reaction led to an increase in FAEE yield from 78.1% in the solvent-free system to 84.4% at the optimal [EtOH]/[FFA] ratio. However, further increase of *tert*-butanol led to lower FAEE yields when compared at the same ethanol content. A sharp increase in FAEE yield was observed during the early phase of incubation, leading to >95% of the maximized conversion yields after 6 h incubation at 50 °C (Fig. 5). CL-PCMC-lipase loading at 20% (w/w) based on FFA was found to be optimal (data not shown). The biocatalyst loading in this study was in the same range (4–50%)

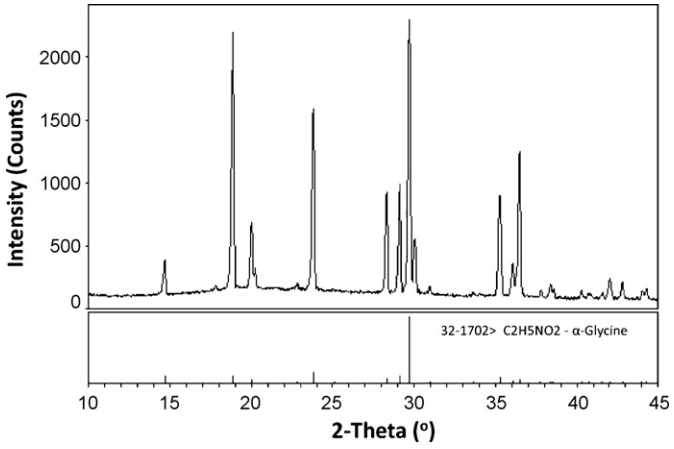

Fig. 3. X-ray diffraction analysis of glycine-based CL-PCMC-lipase. Reference XRD pattern of α-glycine (JCPDS number 32-1702) is shown in the lower panel.

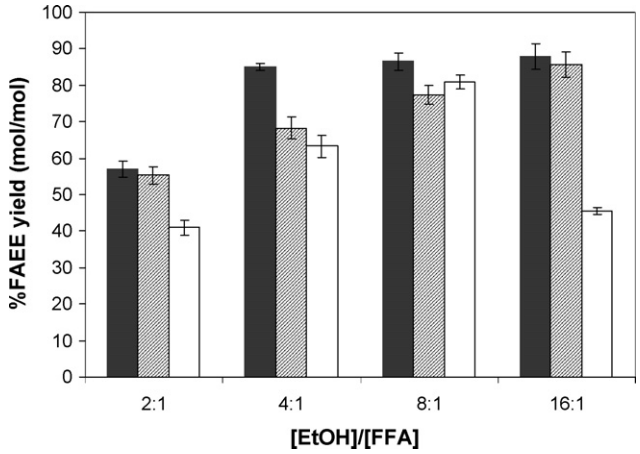

Fig. 4. Effects of nucleophile and co-solvent concentrations on FAEE synthesis. The reactions contained 250 mg of palmitic acid as the substrate with 20% (w/w) CL-PCMC-lipase with varying ethanol and *tert*-butanol ratios to FFA. The reactions were incubated at 50 °C for 6 h. [*t*-BuOH]/[FFA] = 1:1 (black); 2:1 (shaded); and 4:1 (white).

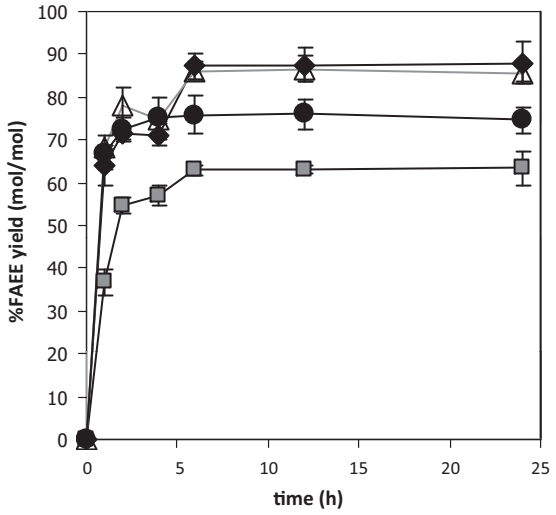

Fig. 5. Reactivity of CL-PCMC-lipase on biodiesel synthesis from FFAs and PFAD. The reactions contained 250 mg of FFAs or PFAD, 4:1 [EtOH]/[FFA] molar ratio, in the presence of 1:1 [*t*-BuOH]/[FFA] molar ratio with 20% (w/w) CL-PCMC-lipase. The reactions were incubated at 50 °C. Substrate: Palmitic acid (diamond); Oleic acid (square); Linoleic (triangle); and PFAD (circle).

as those previously reported using different forms of immobilized lipase on transesterification [10,12,21,22]. The optimal reaction conditions for CL-PCMC-lipase catalyzed reactions were thus at 4:1 [EtOH]/[FFA] in the presence of 1:1 [*t*-BuOH]/[FFA] as the co-solvent with CL-PCMC-lipase loading at 20% (w/w) and incubation at 50 °C for 6 h. The optimal conditions were used for subsequent experiments in this study.

The trend observed of increasing FAEE yield with increasing nucleophile concentration is the opposite to that previously reported for transesterification of refined palm olein using PCMC-lipase [12]. This can be explained by different sensitivity of the biocatalysts to the nucleophile (ethanol) in the reaction, involving deactivation effect on the biocatalyst contact of the lipases with the immiscible polar organic phase [21]. Although a decrease in FAEE yield might be observed at a very high nucleophile and co-solvent ratio, the results shown here suggest an improved ethanol tolerance of CL-PCMC for esterification reactions compared to the conventional PCMCs.

The enhancing effect of *tert*-butanol in the reaction medium has been reported for different forms of immobilized lipases *e.g.* PCMCs and whole-cell biocatalysts for transesterification of triacylglyceride based feedstocks [23–25]. The optimal equimolar ratio of *tert*-butanol and FFA is similar to the previous studies using PCMC-lipase [12] and lower than that for the commercial immobilized lipases and whole-cell biocatalysts in which 1–1.5:1 volume ratio of the co-solvent to oil feedstock was used [23,24]. Addition of *tert*-butanol to the reaction mixture was shown to increase catalytic activity and operational stability of lipases, resulting in increasing conversion yields [23,26]. The activation and stabilization of lipases in esterification could be due to the effects of *tert*-butanol on lipase stabilization from the nucleophile inactivation by linear low molecular weight alcohols [25]. To our knowledge, although the catalysis and stability enhancing effects of *tert*-butanol have been shown for lipase-catalyzed transesterification and whole-cell catalyzed esterification of FFAs [23,27], this study is the first to demonstrate these effects on precipitation-based immobilized lipases in esterification of FFAs on the biodiesel synthesis reaction.

The potential of CL-PCMC-lipase on esterification of FFAs and PFAD was compared with other types of immobilized lipases under the same enzyme loading (20%) and reaction conditions (Fig. 6). CL-PCMC-lipase led to high FAEE yields from palimitic acid (87.1%) and PFAD (81.4%). Ethyl palmitate shared the highest fraction in the esterification product from PFAD in comparison with ethyl oleate and ethyl linoleate, reflecting the FFA composition in PFAD and the biocatalyst reactivity towards different FFAs. The FAEE yields from CL-PCMC-lipase were higher than those using PCMC-lipase prepared on glycine in acetone (75.7% and 67.5% for palmitic and PFAD, respectively), suggesting the additional effects of crosslinking in higher performance of CL-PCMCs and also the widely used immobilized *Candida antarctica* lipase (Novozyme®435) (79.5% and 63.3% for palmitic acid and PFAD, respectively). In contrast, Lipolase 100T led to low FAEE yields from both substrates. PCMC-lipase prepared on K_2SO_4 showed the optimal operational temperature at 45 °C [12] suggesting partial inactivation of PCMC-lipase at 50 °C. The higher product yields from CL-PCMCs could thus be partially due to the improved thermostability of the biocatalyst in comparison with PCMCs by the effect of enzyme molecule crosslinking [13]. Addition of molecular sieve for continuous removal of water from the reaction led to no significant increase in FAEE yields from FFAs and PFAD, which was in contrast to some previous reports in which simultaneous dehydration resulted in significant improved product yields [27,28]. This would suggest the less sensitivity of CL-PCMC-lipase on water activity in esterification of FFAs. In overall, the reactivity of the glycine-based CL-PCMC-lipase in this study was comparable to that of various forms of immobilized lipases for esterification of feedstock

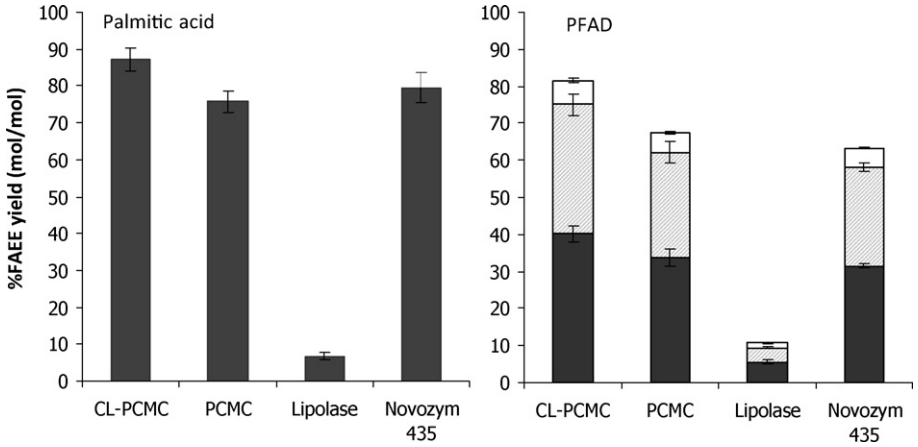

Fig. 6. Comparison of FAEE synthesis using different immobilized lipases. The reactions contained 250 mg of palmitic acid or PFAD, 4:1 [EtOH]/[FFA] molar ratio, in the presence of 1:1 [*t*-BuOH]/[FFA] molar ratio with 20% (w/w) CL-PCMC-lipase. The reactions were incubated at 50 °C for 6 h. CL-PCMC: CL-PCMC-lipase prepared from glycine in acetone; PCMC: PCMC-lipase prepared from glycine in acetone. FAEE products: ethyl palmitate (black); ethyl oleate (shaded); and ethyl linoleate (white).

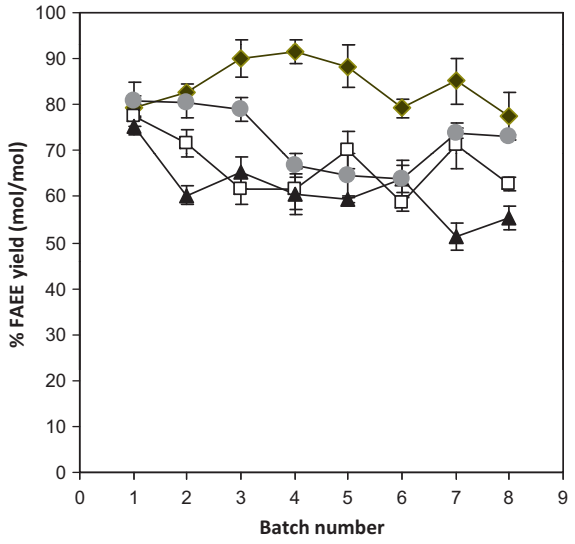

Fig. 7. Stability of CL-PCMC-lipase in consecutive batch reactions. CL-PCMC-lipase was reused in consecutive batch reactions with or without organic solvent treatment. The reactions contained 250 mg of palmitic acid, 4:1 [EtOH]/[FFA] molar ratio, in the presence of 1:1 [*t*-BuOH]/[FFA] molar ratio with 20% (w/w) CL-PCMC-lipase. The reactions were incubated at 50 °C for 6 h. CL-PCMC-lipase was treated by washing with 1 ml of the solvent twice before using in the consecutive batch. No solvent wash treatment (diamond); ethanol (square); *n*-propanol (triangle), and *tert*-butanol (circle).

containing high FFA content *e.g.* soybean oil deodorizer distillate [29] and used palm oil [30], although this cannot be directly compared due to the sensitivity of biocatalysts to the feedstock (*i.e.* type of substrates and contaminants) and reaction conditions. The high conversion yields thus demonstrated the potential of the glycine-based CL-PCMC-lipase as an economical heterogeneous biocatalyst for biodiesel production by esterification of FFAs in feedstocks.

3.3. Reusability of CL-PCMC-lipase

The reusability of CL-PCMC-lipase was studied by analyzing the conversion efficiency after consecutive batch cycles under the optimal reaction conditions (Fig. 7). CL-PCMC-lipase showed high stability in esterification of palmitic acid with no significant alteration in FAEE yield for at least 8 consecutive batch processes with the average product yields of 84.8% ± 5.0%. Treatment by organic solvents with different polarities (ethanol, *n*-propanol and *tert*-butanol) led to no improvement on FAEE yields, leading to 62.7, 55.3, and 73.0% FAEE yield in batch 8. The effect of *tert*-butanol on the biocatalyst stability was different to the K_2SO_4-based PCMC-lipase, in which *tert*-butanol treatment led to improved stability of the biocatalyst in consecutive batches of palm olein transesterification [12]. This could be due to the nucleophilic deactivation effect of short chain alcohols, particularly ethanol and propanol on the biocatalyst stability. The result thus suggested the potential of recycling CL-PCMC-lipase in further consecutive batch process development with no additional organic solvent treatment.

4. Conclusion

Biocatalytic synthesis by CL-PCMC-lipase is considered a promising approach for biodiesel production from feedstocks containing high FFA contents. The optimized process led to high product yields comparable to those previously reported for acid-catalyzed [31], thermocatalytic [4] and whole-cell biocatalytic methods [25]; however, with its key advantages over the existing methods, including mild operating conditions and low catalyst preparation cost. The use of glycine as the core matrix for precipitation-based immobilized enzyme was reported, suggesting the potential on using glycine as the core matrix component for preparation of high performance CL-PCMCs for catalysis in non-aqueous systems. The biocatalytic process developed in this study thus provides a promising approach for production of biodiesel from inexpensive feedstocks with high FFA contents. Further development of the CL-PCMC-lipase based processes would lead to an improvement on the process economics of biodiesel industry.

Acknowledgements

This project was supported by the Discovery Based Development Grant from the National Science and Technology Development Agency and Thailand Research Fund. Raita M. was supported by the Royal Golden Jubilee Scholarship. Manuscript proofreading by Dr. Philip James Shaw is appreciated. The authors also would like to thank Ms. Suphakan Kijamnajsuk, National Center for Metal and Material Technology for XRD analysis.

References

[1] J. Van Gerpen, Fuel Proc. Technol. 86 (2005) 1097–1107.
[2] P. Mongkolbovornkij, V. Champreda, W. Sutthisripok, N. Laosiripojana, Fuel Proc. Technol. 91 (2010) 1510–1516.
[3] D. Yujaroen, M. Goto, M. Sasaki, A. Shotipruk, Fuel 88 (2009) 2011–2016.
[4] A. Petchmala, N. Laosiripojana, B. Jongsomjit, M. Goto, J. Panpranot, O. Mekasuwandumrong, A. Shotipruk, Fuel 89 (2010) 2387–2392.
[5] A. Robles-Medina, P.A. González-Morino, L. Esteban-Cerdán, E. Molina-Grima, Biotechnol. Adv. 27 (2009) 398–408.
[6] Y. Shimada, Y. Watanabe, A. Sugihara, Y. Tominaga, J. Mol. Catal. B: Enzym. 17 (2002) 133–142.
[7] S. Shah, A. Sharma, M.N. Gupta, Anal. Biochem. 351 (2006) 207–213.
[8] T.S. Lee, J.D. Vaghjiani, G.J. Lye, M.K. Turner, Enzym. Microb. Technol. 26 (2000) 582–592.
[9] M. Kreiner, B.D. Moore, M.C. Parker, Chem. Commun. (2001) 1096–1097.
[10] S. Shah, A. Sharma, D. Varandani, B. Mehta, M.N. Gupta, J. Nanosci. Nanotechnol. 7 (2007) 2157–2160.
[11] V. Kumari, S. Shah, M.N. Gupta, Energy Fuels 21 (2007) 368–372.
[12] M. Raita, V. Champreda, N. Laosiripojana, Proc. Biochem. 45 (2010) 829–834.
[13] S. Shah, A. Sharma, M.N. Gupta, Biocatal. Biotrans. 26 (2008) 266–271.
[14] M. Kreiner, M.C. Parker, Biotechnol. Bioeng. 87 (2004) 24–33.
[15] J.C. Wu, J.X. Yang, S.H. Zhang, Y. Chow, M.M.R. Talukder, W.J. Choi, Biocatal. Biotrans. 27 (2009) 283–289.
[16] S. Murdan, S. Somavarapu, A.C. Ross, H.O. Alpar, M.V. Parker, Int. J. Pharm. 296 (2005) 117–121.
[17] A. Khosravani, M.-C. Parker, R. Parton R., J. Coote, Vaccine 25 (2007) 4361–4367.
[18] N. Harper, M. Dolman, B.D. Moore, P.J. Halling, Chem. Eur. J. 6 (2000) 1923–1929.
[19] S. Shah, M.N. Gupta, Bioinorg. Med. Chem. Lett. 17 (2007) 921–924.
[20] K. Solkanki, M.N. Gupta, Bioinorg. Med. Chem. Lett. 21 (2011) 2934–2936.
[21] E. Hernández-Mártin, C. Otero, Bioresour. Technol. 99 (2008) 277–286.
[22] A. Salis, M. Pinna, M. Monduzzi, V. Solinaz, J. Mol. Catal. B: Enzym. 54 (2008) 19–26.
[23] L.L. Li, W. Du, D.H. Liu, J. Mol. Catal. B: Enzym. 43 (2006) 58–62.
[24] L. Li, W. Du, D. Liu, Proc. Biochem. 43 (2007) 1481–1485.
[25] W. Du, D. Liu, L. Li, D. Dai, Biotechnol. Prog. 23 (2007) 1087–1090.
[26] D. Royon, M. Daz, G. Ellenrieder, S. Locatelli, Bioresour. Technol. 98 (2007) 648–653.
[27] W. Li, W. Du, D. Liu, Energy Fuels 22 (2008) 155–158.
[28] L. Wang, W. Liu, D. Liu, L. Li, N. Dai, J. Mol. Catal. B: Enzym. 43 (2006) 29–32.
[29] M.S. Souza, E.C.G. Aguieiras, M.A.P. Da Silva, M.A.P. Langone, Appl. Biochem. Biotechnol. 154 (2009) 253–267.
[30] K. Tongboriboon, B. Cheirsilp, A. H-Kittikun, J. Mol. Catal. B: Enzym. 67 (2010) 52–59.
[31] S. Chongkhong, C. Tongurai, P. Chetpattananondh, C. Bunyakan, Biomass. Bioener. 31 (2007) 563–568.