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Immobilization of Lipase onto *Cyperus Papyrus* L. for Biodiesel Production by Transesterification and Hydrolysis-Esterification

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Abstract

Lipase of *Candida rugosa* was immobilized on biomass support, plant fiber from *Cyperus papyrus* L., for biodiesel synthesis. The two immobilization techniques investigated in this study were physical adsorption and covalent binding with 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). Results showed that the prepared immobilized lipase by physical adsorption with adding heptane presented higher protein loading, lipase activity and degree of immobilization than that by physical adsorption in phosphate buffer solution and by covalent binding. This immobilized lipase was further applied as biocatalyst for biodiesel synthesis by transesterification and hydrolysis-esterification. The results showed that this immobilized lipase preferred to hydrolyze triglyceride in palm oil to be fatty acid in water as medium. Then, the obtained fatty acid could be a good substrate to react with alcohol for biodiesel synthesis by esterification route. So, the biodiesel synthesis yield by hydrolysis-esterification was higher than transesterification. Nevertheless, the results revealed that bioethanol was found to be a better substrate than methanol for biodiesel synthesis via enzymatic hydrolysis-esterification.

Key words: *Cyperus papyrus* L., *Candida rugosa* lipase, Immobilization, Biodiesel, Transesterification, Hydrolysis-esterification

Introduction

Biodiesel, monoalkyl esters of vegetable oils or animal oils, is viewed as new alternative renewable, non-toxic, biodegradable and clean energy source. It does not contribute to global warming due to its large reduction of CO_2 emission compared with petroleum-based oil. Enzymatic process using lipase as catalyst for biodiesel production instead of acid and alkaline has attracted much attention because of its easy recovery of biodiesel and glycerol, its lower energy consumption, and its non-toxic and biodegradable characteristics [(1)]. Due to relatively high cost of lipase, this process could not be economically acceptable unless high stable forms of lipase are available. To solve this problem, lipase should be immobilized so that it can be recovered easily from the product and can also be reused. Many immobilized lipases are commercially available; however, their costs are relatively high due to the usage of the expensive support material to immobilize onto lipase. Therefore, cheaper supports such as clays, silica gels, and glasses are used as the alternative adsorbents to the expensive polymeric resins [(2-4)]. Other attractive supports are plant fibers, especially cellulosic fibers because of their accessibility, cheapness, hydrophilic character in nature, great number of hydroxyl groups on the surface capable of chemical reaction [(5)]. Moreover, most plant fibers possess a large

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number of pores. Lipase, thus, has a possibility to immobilize onto plant fibers through simple adsorption. The adsorption technique is simpler and less expensive than other techniques such as cross-linking or entrapment techniques, and high catalytic activity may be retained [4, 6].

In this study, plant fiber from *Cyperus papyrus* L. which is abundant in Thailand, grows easily in nature, and is of very low cost, was used as biomass support to immobilize *Candida rugosa* lipase for biodiesel synthesis. The two types of immobilization techniques used were physical adsorption techniques using heptane (Im-ADHEP), and phosphate buffer solution (Im-ADBUF) as media and covalent binding techniques using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (Im-CB). Protein loading, lipase activity and degree of immobilization of immobilized lipase were subsequently investigated. The catalytic activities of immobilized lipase in transesterification and hydrolysis-esterification reaction using palm oil and methanol or bioethanol as substrate were further examined as a potential biocatalyst for biodiesel synthesis.

Materials and Experimental Procedures

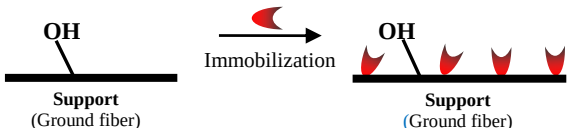
Materials

Candida rugosa lipase powder was purchased from Sigma-Aldrich. Plant fibers, *Cyperus papyrus* L., from Nakhon Ratchasima Province, Thailand, were used as support. It was ground into powder using Pulverizer model T15. Palm oil from Morakot Industries, Thailand, was used as substrate triglyceride. Heptane and phosphate buffer used as media for lipase immobilization were supplied by Labscan, Thailand, and Merck, Germany, and were of analytical grade. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) used for the activation of hydroxyl group of *Cyperus papyrus* L. was purchased from Sigma-Aldrich.

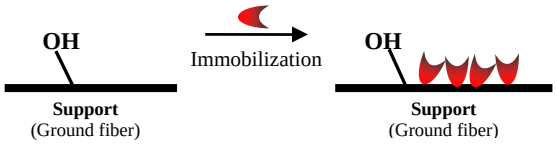
Lipase Immobilization on Cyperus papyrus L.

The two techniques of lipase immobilization used in this study were physical adsorption and covalent binding. A schematic illustration of the different techniques for lipase immobilization is shown in Figure 1 (a) to (c).

(a) Physical adsorption (in heptane)



(b) Physical adsorption (in phosphate buffer solution)



(c) Covalent binding with EDC (in phosphate buffer solution)

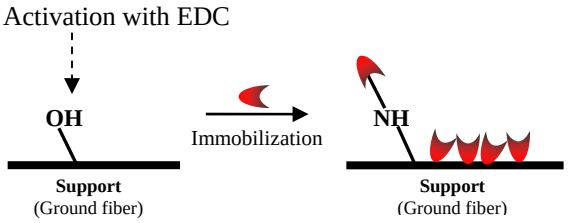


Figure 1. Schematic illustration of lipase immobilization techniques.

lipase

Physical Adsorption in Heptane (Im-ADHEP)

0.25 g of ground fiber, 0.12 g of lipase and 5 ml of heptane were mixed and gently stirred for 12 hours at room temperature. The immobilized lipase was washed with 5 ml of heptane 6 times. It was then left to dry at room temperature in a desiccator for 1 night.

Physical Adsorption in Phosphate Buffer Solution (Im-ADPB)

0.25 g of ground fiber, 5 ml of lipase solution (0.1 g/ml) were mixed and magnetically stirred at 300 rpm for 7 hours at room temperature. The immobilized lipase was washed with 5 ml of phosphate buffer solution (pH 7) 6 times. It was then left to dry at room temperature in a desiccator.

Covalent Binding Using EDC as an Activator (Im-CB)

0.25 g of ground fiber was first activated by 0.75% (w/v) EDC which was dissolved in 5 ml of phosphate buffer solution (pH 6) for 10 minutes at 25°C. The reaction mixture was then washed with distilled water and followed by mixing with 5 ml of lipase solution (0.1 g/ml) and stirred at 360 rpm for 1 hour at room temperature. The immobilized lipase was then washed with 5 ml of phosphate buffer solution (pH 7) 6 times and left to dry at room temperature in a desiccator.

Morphology of Immobilized Lipase

A scanning electron microscope (SEM) (Jeol JSM-5800 LV) under accelerated voltage of 15 kV was used to examine the morphology of *Cyperus papyrus* L. before and after immobilization.

Biodiesel Synthesis

In this study, the catalytic activities of immobilized lipase in transesterification and hydrolysis-esterification reaction using palm oil as substrate and methanol or bioethanol for biodiesel synthesis were studied. The biodiesel synthesis reaction is shown in Figure 2 (a) to (b).

Transesterification

In transesterification, the stepwise methanolysis of palm oil was conducted as follows: 1 molar of palm oil was first mixed with the immobilized lipase or free lipase and stirred for 30 minutes at room temperature. According to Shimada et al, the molar ratio of palm oil:methanol chosen was 1:3 [7]. Thus, 3 molar of methanol were then added to the palm oil by 3 steps feeding and stirred at 600 rpm for 24 hours at 40°C. After the transesterification reaction, the products were taken from the reaction mixture and allowed to separate by centrifugation at 13,000 rpm for 30 minutes. The upper part of samples was then taken to analyze the fatty acid alkyl ester by high performance liquid chromatography (HPLC, Shimadzu LC-20A series).

Hydrolysis-Esterification

Hydrolysis of Palm Oil

Palm oil was hydrolyzed by mixing 1 molar of palm oil and the solution of immobilized lipase or free lipase. Then, 300 μl of distilled water were added to the mixture and continuously stirred at 600 rpm for 24 hours at 40°C.

Esterification

In esterification, the stepwise methanolysis of palm oil was conducted. According to Shimada et al, the molar ratio of palm oil:methanol chosen was 1:3 [7]. Thus, 3 molar of methanol or bioethanol were then added to the hydrolyzed palm oil by 3 steps feeding and stirred at 600 rpm for 24 hours at 40°C. After the esterification reaction, the products were taken from the reaction mixture and allowed to separate by centrifugation at 13,000 rpm for 30 minutes. The upper part was then taken to analyze the fatty acid alkyl ester by high performance liquid chromatography (HPLC, Shimadzu LC-20A series) [8].

Determination of Protein Loading

Protein concentration was spectrophotometrically determined by UV spectrophotometer (ANTHOS Zenyth 200 Microplate Spectrophotometer) according to Bradford method using bovine serum albumin (BSA) as the standard [8].

Determination of Lipase Activity

Activity of the free and immobilized lipase was determined using 0.5% (w/v) *p*-nitrophenyl palmitate (*p*-NPP) in ethanol as substrate. The increase in absorbance at 410 nm caused by the release of *p*-nitrophenol was measured by UV spectrophotometer (ANTHOS Zenyth 200 Microplate Spectrophotometer). One unit (U) of lipase activity was defined as the amount of enzyme necessary to hydrolyze 1 nanomol/min of *p*-NPP under the experimental conditions.

Results and Discussion

Morphology of Immobilized Lipase

The SEM images of free lipase, ground *Cyperus papyrus* L. fiber and immobilized lipase by physical adsorption (Im-ADHEP and Im-ADPB) and by covalent binding (Im-CB) are shown in Figure 3 (a) to (e). The presence of lipase on the surface and also inside the pore of fiber is seen in the SEM images of the Im-ADHEP, Im-ADPB as well as of the Im-CB. The aggregation of the lipase particles on the fiber surface was observed when they were immobilized by physical adsorption in phosphate buffer solution and by covalent binding with EDC, whereas the lipase particles were well dispersed inside the pores of fiber which was similar to the free lipase when they were immobilized by physical adsorption in heptane.

The Efficiency of Lipase Immobilization

The efficiency of lipase immobilization using physical adsorption technique with different media (Im-ADHEP and Im-ADPB) and covalent binding technique (Im-CB) is shown in Table 1. Best results were found when the immobilization was performed by physical adsorption in the presence of heptane, 83.2 μg/g-fiber of protein loading, 10.1 U/g-fiber of lipase activity and 93.4% of degree of immobilization can be obtained. This may be explained by the conformation change of lipase molecule according to the medium polarity [9]. It is generally acknowledged that lipases are interface-active enzymes with lipophilic domains and can adopt both open and close conformations. Lipophilic interactions between the hydrophobic parts of the lipase and substrate or organic solvent could fix the entrapped enzyme in a higher conformation and enhance activity [10]. In buffer solution the more hydrophilic amino acids of lipase become exposed to the media, and the enzyme tends to fold. Nevertheless, in a medium with low polarity, lipophilic domains may interact with heptane. This effect seems to help induce conformational changes of lipase which result in the active form. Therefore, this would allow free access of the substrate to the active site of the immobilized lipase and increase activity of lipase via 'interphase activation mechanism' [11, 12]. Moreover, the most inferior result was found when the immobilization was performed by covalent binding. This may be due to the extensive covalent bonding between lipase and support through EDC activation which may result in a distortion of the enzyme structure (i.e. active site conformation). With this distortion, the accessibility and accommodation of the substrate may be reduced. This affects the retention of biological activity [13,14].

Table 1. Protein loading, activity and degree of immobilization of immobilized lipase by physical adsorption and by covalent binding.

Immobilization techniques	Protein loading (μg/g-fiber)	Lipase activity (U/g-fiber)	Lipase activity change (%)	Degree of immobilization (%)
Im-ADHEP[a]	83.2	10.1	-11.2	93.4
Im-ADPB[b]	80.5	7.1	-61.7	82.8
Im-CB[b]	78.7	6.2	-66.6	76.2

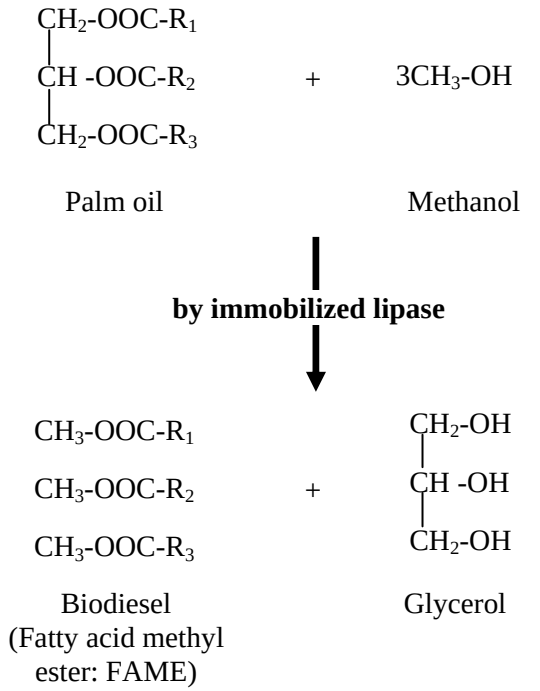
[a] Lipase activity of free lipase is 11.40 U/ml
[b] Lipase activity of free lipase is 18.63 U/ml

Conversion of Alkyl Ester (biodiesel) Produced by Lipase-catalyzed Reaction

Table 2 shows the % conversion of alkyl ester produced by transesterification and hydrolysis-esterification using palm oil and methanol or bioethanol as substrate. Results indicated that all lipases (free lipase and immobilized lipase prepared from different techniques) can catalyze transesterification and hydrolysis-esterification for biodiesel synthesis, but at different conversion. Best results were found when Im-ADHEP was used by both transesterification and hydrolysis-esterification. It is observed that Im-ADHEP-catalyzed transesterification of palm oil and methanol yielded a conversion of methyl ester (19.3%) similar to the conversions of the free lipase. However, hydrolysis-esterification yielded a higher conversion of methyl ester (48.8%) than that of transesterification. This can be explained by the fact that the specific activity of lipase from *Candida rugosa* prefers, in the first step, water to hydrolyze triglyceride in palm oil to be fatty acids; subsequently, fatty acids became an appropriate substrate that can react with alcohol for biodiesel synthesis. However, this immobilized enzyme was not appropriate to catalyze the transesterification for biodiesel synthesis directly [15]. In spite of this, if bioethanol was used instead of methanol, a higher conversion of ethyl ester (51.3%) was obtained. The result

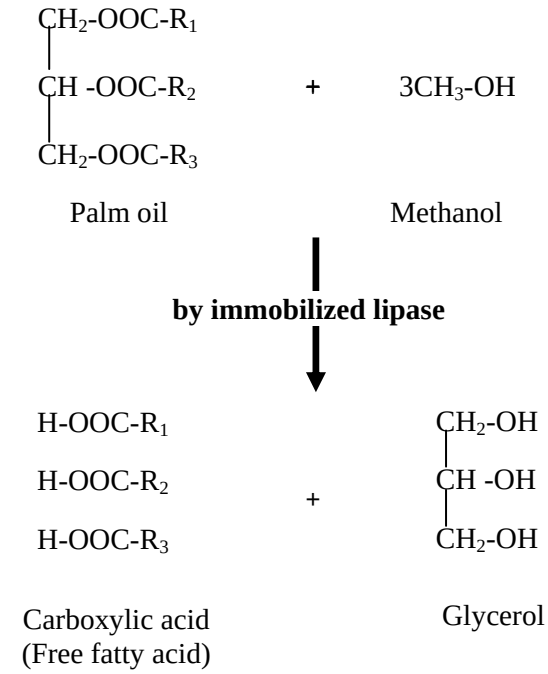
showed that using bioethanol as the substrate instead of methanol leads to the non-toxic and environmentally friendly process.

(a) Transesterification



(b) Hydrolysis-esterification

Hydrolysis



Esterification

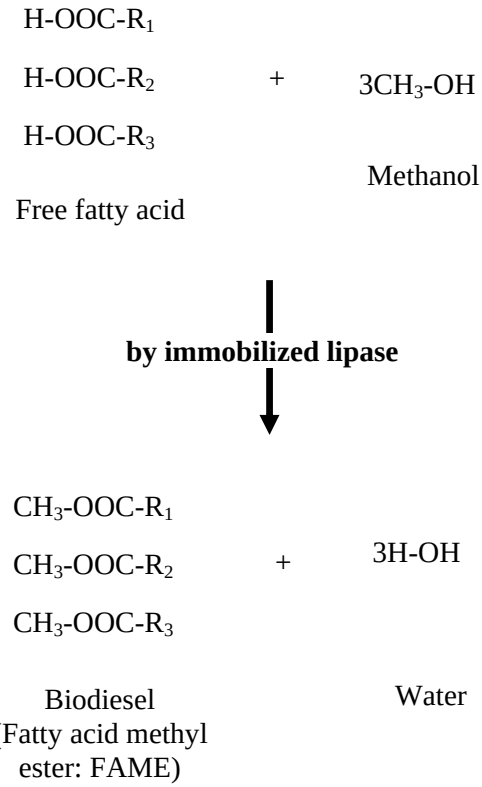


Figure 2. The schematic reactions of biodiesel synthesis via transesterification (a); and hydrolysis-esterification (b).

Table 2. Conversion of alkyl ester (biodiesel) produced by different types of immobilized lipase-catalyzed reaction.

Types of immobilized lipase	% conversion of biodiesel (Alkyl ester)		
	Transesterification (methanol as substrate)	Hydrolysis-esterification	
		Methanol as substrate	Bioethanol as substrate
Free lipase	20.2	23.4	24.5
Im-ADHEP	19.3	48.8	51.3
Im-ADPB	12.9	10.6	17.2
Im-CB	7.5	2.6	21.6

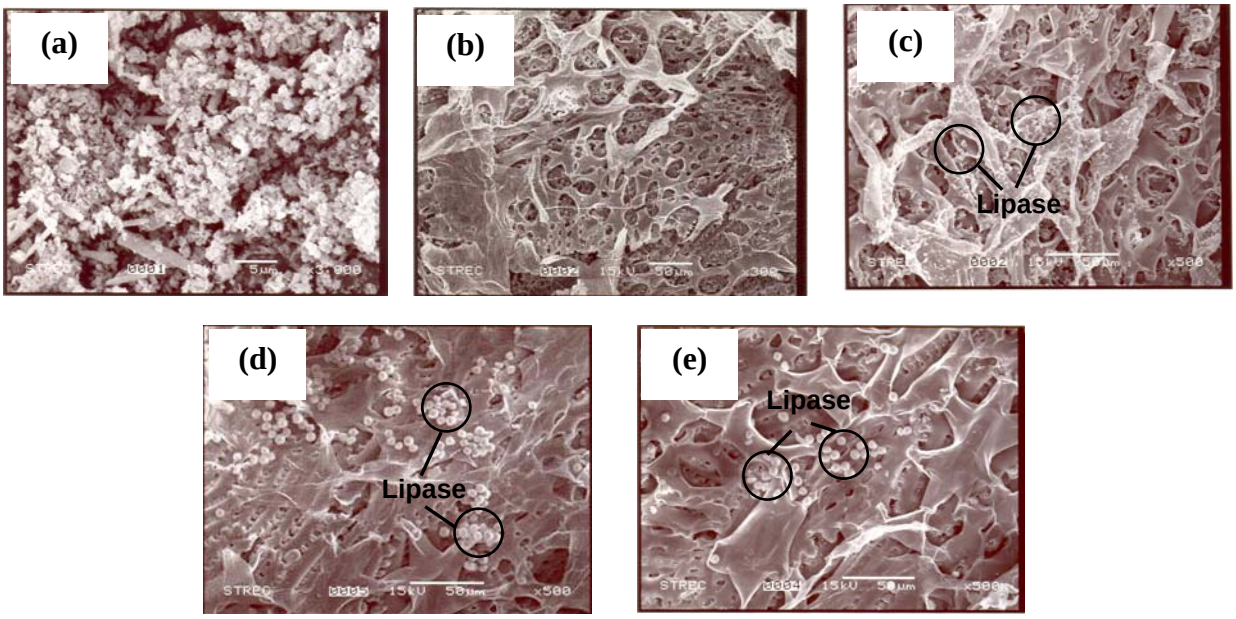


Figure 3. SEM images of free lipase (a); ground *Cyperus papyrus* L. fiber (b); immobilized lipase by physical adsorption in heptane, Im-ADHEP (c); immobilized lipase by physical adsorption in phosphate buffer solution, Im-ADPB (d); and by covalent binding, Im-CB (e) onto ground *Cyperus papyrus* L.

Conclusion

Immobilization of *Candida rugosa* lipase onto biomass support, plant fiber from *Cyperus papyrus* L. by physical adsorption with adding heptane (Im-ADHEP) provided higher efficiency of immobilization than that of physical adsorption with adding phosphate buffer (Im-ADPB) and by covalent binding with EDC (Im-CB). In terms of biodiesel synthesis, Im-ADHEP-catalyzed transesterification of palm oil and methanol yielded a conversion of methyl ester similar to those of the free lipase, whereas hydrolysis-esterification yielded a higher conversion of methyl ester than that of transesterification. Furthermore, the higher conversion of ethyl ester was obtained if bioethanol was used instead of methanol. This suggested that using bioethanol as the substrate for biodiesel synthesis instead of methanol leads to the non-toxic and environmentally friendly process.

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