รายงานวิจัยฉบับสมบูรณ์

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Biodiesel Production from Palm Oil by Immobilized Lipase : Kinetic Modeling and Optimization

โดย ผศ. ดร. เบญจมาส เชียรศิลป์

กรกฎาคม 2551

สัญญาเลขที่ MRG4980088

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บทคัดย่อ

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ระยะเวลาโครงการ: **2 ปี**

ในการผลิตแฟตตี้เอซิดเอทิลเอสเทอร์ (fatty acid ethyl ester, FAEE) จากน้ำมันปาล์ม พบว่าเอนไซม์ไลเปสทางการค้าจากเชื้อ *Pseudomonas* sp. (lipase PS) เหมาะสมที่สุด โดยสภาวะที่เหมาะสมในการตรึงรูปเอนไซม์ lipase PS บนตัวตรึง Accurel คือความเข้มข้นเอนไซม์ 50 ยูนิตต่อมล. เวลาในการตรึง 30 นาที เอนไซม์ lipase PS ตรึงรูปที่ได้มีกิจกรรม 0.258 ยูนิตต่อมก. และมีประสิทธิภาพการยึดเกาะเท่ากับร้อยละ 83.79 สภาวะที่เหมาะสมในการผลิต FAEE จากน้ำมันปาล์มคือ ปริมาณเอนไซม์ตรึงรูปร้อยละ 4 และที่อุณหภูมิ 45 องศาเซลเซียส ปริมาณน้ำที่เติมในปฏิกิริยาที่เหมาะสมสำหรับการผลิต FAEE จากน้ำมันปาล์มบริสุทธิ์ น้ำมันปาล์มดิบ และน้ำมันปาล์มใช้แล้ว คือร้อยละ 10, 2 และ 0 ตามลำดับ และปริมาณสัดส่วนเอทานอลต่อน้ำมันปาล์มที่ 3:1 ให้ผลผลิต FAEE สูงสุดเท่ากับร้อยละ 97, 94 และ 96 สำหรับน้ำมันปาล์มบริสุทธิ์ น้ำมันปาล์มดิบ และน้ำมันปาล์มใช้แล้ว ตามลำดับ ที่เวลา 48 ชม. นอกจากนี้ยังพบว่าการแบ่งเติมแอลกอฮอล์ 3 ครั้งในการผลิตจะทำให้อัตราการผลิต FAEE สูงขึ้น

ในการศึกษาทางจลนพลศาสตร์ งานวิจัยนี้ได้นำเสนอแบบจำลองของปฏิกิริยาทรานส์เอสเทอริฟิเคชั่นของกรดไขมันในน้ำมันปาล์มกับเอทานอลโดยใช้เอนไซม์ไลเปสตรึงรูป 3 แบบจำลอง โดยแบบจำลองทั้ง 3 สามารถแสดงผลของสับสเตรทและผลิตภัณฑ์ในปฏิกิริยาทรานส์เอสเทอริฟิเคชั่นได้ตลอดการทดลอง จากการเปรียบเทียบผลการทดลองและผลที่ได้จากการคำนวณ พบว่าสมการแบบจำลองที่สร้างจากสมมติฐานที่ว่าเอทานอลเข้าทำปฏิกิริยาแอลกอฮอไลซิสโดยตรงกับน้ำมันปาล์มจะให้ผลการคำนวณใกล้เคียงกับผลการทดลองมากที่สุด และจากการเลียนแบบจำลองพบว่าการเพิ่มความเข้มข้นเอทานอลจะทำให้อัตราการผลิตเริ่มต้นและผลผลิตของ FAEE เพิ่มขึ้น และทำให้ความเข้มข้นสุดท้ายของกรดไขมันอิสระลดลง ในขณะที่ความเข้มข้นของเอทานอลที่ต่ำจะทำให้เกิดเป็นกรดไขมันอิสระในปริมาณที่สูง

ในการผลิต FAEE แบบต่อเนื่องในถังปฏิกรณ์แบบแพคเบด พบว่าผลผลิตของ FAEE สูงขึ้นเมื่อลดอัตราการไหลของสับสเตรท และพบว่าที่อัตราการไหลของสับสเตรท 0.29 กรัมต่อ

นาที จะให้ผลผลิต FAEE ที่ร้อยละ 38.25 และให้ผลผลิต FAEE ต่อเวลาสูงสุดที่ 0.111 กรัมต่อนาที นอกจากนี้ยังพบว่ากระบวนการผลิตแบบต่อเนื่องสามารถคงผลผลิตเฉลี่ยได้ที่ร้อยละ 34 เป็นเวลา 90 ชั่วโมง โดยให้อัตราการผลิต FAEE เท่ากับ 142 กรัมต่อวัน

คำหลัก: ไบโอดีเซล เอนไซม์ไลเปสตรึงรูป น้ำมันปาล์ม การเลียนแบบจำลอง

Abstract

Project Code: MRG4980088

Project Title: Biodiesel Production from Palm Oil by Immobilized Lipase : Kinetic Modeling and Optimization

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Project Period: 2 years

A commercial lipase from *Pseudomonas* sp. (lipase PS) was the suitable enzyme for synthesis of fatty acid ethyl ester (FAEE) from palm oil. The optimum condition for immobilization of lipase PS on Accurel was enzyme concentration of 50 U/ml and immobilization time for 30 min. The activity of immobilized lipase PS was 0.258 U/mg and immobilized yield was 83.79%. The optimum conditions for FAEE production from palm oil were found to be 4% of immobilized lipase PS and at temperature 45°C. The optimal water additions for FAEE production from refined, crude and used palm oils were 10%, 2% and 0%, respectively, based on oil weight. The molar ratio of ethanol to palm oil at 3:1 gave the highest FAEE yield of 97%, 94% and 96% for refined, crude and used palm oil, respectively, at 48 h. Moreover, three-step addition of alcohol has improved the productivity of FAEE.

For kinetic study, three kinetic models of the transesterification of palm oil fatty acids to ethanol using an immobilized lipase were developed. The models are able to account for the effects of substrates and products involved in the transesterification throughout the entire reaction. There was a good agreement between experimental results and those predicted by the proposed model equations in which ethanol was assumed to be involved directly in an alcoholysis reaction with palm oil. From the proposed model equations, the simulation results show that increasing the initial ethanol concentration produces an increase in the initial production rate and yield of FAEE and lowers the final concentration of free fatty acid whereas lower ethanol concentration led to a higher final concentration of free fatty acid.

In the continuous production of FAEE in a packed-bed reactor, the yield of FAEE increased with decreasing in the flow rate of substrate. At the substrate flow rate of 0.29 g/min gave FAEE yield of 38.25 % and the highest productivity of FAEE of 0.111 g/min. Furthermore, the long term operation of the continuous process gave the average yield of FAEE at 34% for 90 h with the productivity of 142g FAEE/day.

Keywords: biodiesel, immobilized lipase, palm oil, modeling

EXECUTIVE SUMMARY

Biodiesel-fatty acid alkyl esters of natural fats and oils have attracted much research interest. They are renewable while the availability of petroleum chemicals is finite. Fatty acid alkyl esters are produced by transesterification of triglycerides with alcohols preferentially methanol and ethanol. Biodiesel from palm oil can be domestically produced, offering the possibility of reducing petroleum imports. Recently, utilization of lipase in the production of fatty acid alkyl esters has been shown to be effective. Lipase-catalyzed synthesis reaction selectivity is high and lipase can be immobilized in the support material, which will allow for its recovery and reuse. Several researchers have investigated biodiesel production from palm oil using immobilized *Candida antarctica* lipases. However, the bottleneck of lipase-catalyzed biodiesel production is the high price of the enzyme. Since the price of *Candida antarctica* lipase is relatively expensive and far from practical, the study of other low-cost lipases for biodiesel production from palm oil is needed. In addition, in order to identify the optimal conditions for the lipase catalyzed transesterification reaction, it is essential to understand the kinetics of this reaction. Also the clarification of the mechanisms and the corresponding rate expressions of the transesterification reactions have not been conducted. To elucidate the mechanism of transesterification of two substrates, namely triacylglycerol and alcohol, computer simulation could be a useful tool to independently assess the effects of hypothetical changes in the concentrations of each substrate.

The aim of this study was to employ enzymatic method in industrial production of biodiesel from palm oil. Firstly, the synthesis of fatty acid ethyl esters (FAEE) from palm oil by various lipases in solvent-free system was investigated. Among four commercial lipases from *Pseudomonas* sp. (lipase PS), *Pseudomonas fluorescens* (lipase AK), *Candida rugosa* (lipase AY) and *Rhizopus delemar* (lipase D) were screened for production of fatty acid ethyl esters (FAEE) from palm olein. Lipase PS was the only suitable enzyme for synthesis of FAEE from palm oil.

For industrial application, immobilization of lipase PS was optimized. Accurel EP-200 was used as support to immobilize lipase PS. The optimum condition for immobilization was enzyme concentration of 50 U/ml and immobilization time for 30 min. The activity of immobilized lipase PS was 0.258 U/mg and immobilized yield was 83.79%.

Optimal reaction parameters for synthesis of fatty acid alkyl esters from palm oil were also determined. The optimum condition for FAEE production from 5 g of palm olein was found to be 50U of immobilized lipase PS, 0.5 ml of water addition and at temperature 45°C. The optimal molar ratios of palm olein to ethanol and methanol for production of FAEE and fatty acid methyl esters (FAME), respectively, were the same at 1:3. Under these conditions, the yields of 61% FAEE and 60% FAME were obtained at 6 h. Moreover, three-step addition of alcohol has improved yields of FAEE and FAME up to 76% and 75%, respectively.

The kinetics of transesterification of palm oil and ethanol for fatty acid ethyl ester production was investigated. A simple model based on Ping-Pong Bi Bi was proposed to describe the kinetics of the transesterification and hydrolysis reactions. Three kinetic models of the transesterification of palm oil fatty acids to ethanol using an immobilized lipase were developed. The models differ from one another with respect to the rate-limiting step and the point at which the ethanol molecule becomes involved in the reaction. The kinetic parameters were estimated by fitting experimental data of the transesterification of palm oil with various ethanol concentrations. The models are able to account for the effects of substrates and products involved in the transesterification throughout the entire reaction. There was a good agreement between experimental results and those predicted by the proposed model equations in which ethanol was assumed to be involved directly in an alcoholysis reaction with palm oil. Furthermore, the calculated results show that the rate constants for alcoholysis of palm oil with ethanol are much higher than those for the hydrolysis reaction. From the proposed model equations, the effects of ethanol concentration on the initial production rates and yields of FAEE and free fatty acids were simulated. The simulation results show that increasing the initial ethanol concentration produces an increase in the initial production rate and yield of FAEE and lowers the final concentration of free fatty acid whereas lower ethanol concentration led to a higher final concentration of free fatty acid.

Finally, in the continuous production of FAEE in a packed-bed reactor, the yield of FAEE increased with decreasing in the flow rate of substrate. At the substrate flow rate of 0.29 g/min gave FAEE yield of 38.25 % and the highest productivity of FAEE of 0.111 g/min. Furthermore, the long term operation of the continuous process gave the average yield of FAEE at 34% for 90 h with the productivity of 142g FAEE/day.

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CHAPTER 1 Introduction

1.1 Importance and Motivation of This Research

Biodiesel-fatty acid alkyl esters of natural fats and oils have attracted much research interest. They are renewable while the availability of petroleum chemicals is finite. Fatty acid alkyl esters are produced by transesterification of triglycerides with alcohols preferentially methanol and ethanol. Biodiesel from palm oil can be domestically produced, offering the possibility of reducing petroleum imports. Industrial practice for production of biodiesel from palm oil is typically produced by alkaline or acid catalyzed transesterification (Krisnangkura and Simamaharnnop, 1992; Crabbe et al., 2001; Kalam and Masjuki, 2002). However, the undesired side reaction, saponification, occurs and an extra separation step to remove the homogeneous catalysis is required, thus reflecting the high cost of production.

Recently, utilization of lipase in the production of fatty acid alkyl esters has been shown to be effective. In the transesterification of soybean oil, 80-90% conversion of esters was obtained using *Rhizopus oryzae* lipase solution (Kaieda et al., 1999). Lipase-catalyzed synthesis reaction selectivity is high and lipase can be immobilized in the support material, which will allow for its recovery and reuse. Transesterifications of various plant oils using immobilizied lipases have been studied (Dossat et al., 2002: Soumanou and Bornscheuer, 2003; Du et al., 2004; Noureddini et al., 2005; Li et al., 2006). Several researchers have investigated biodiesel production from palm oil using immobilized lipases. Abigor et al. (2000) studied the lipase-catalyzed transesterification of palm kernel oil with different alcohols using lipase PS-30. Talukder et al. (2006) used *Candida antarctica* lipase to produce methyl esters from palm oil in presence and absence of organic solvent. However, the bottleneck of lipase-catalyzed biodiesel production is the high price of the enzyme. Since the price of *Candida antarctica* lipase is relatively expensive and far from practical, the study of other low-cost lipases for biodiesel production from palm oil is needed. Kaieda et al. (2001) reported that lipases from a number of microorganisms are able to catalyze methanolysis with appropriate water and methanol contents in the reaction mixture. Lipase that is relatively resistant to methanol, such as that from *Pseudomonas cepacia*, will be advantageous in low water content, which is desirable to attain a high reaction rate of transesterification.

In addition, in order to identify the optimal conditions for the lipase catalyzed transesterification reaction, it is essential to understand the kinetics of this reaction. Until now all kinetic mechanisms on lipase catalytic reactions have been mostly based on the hydrolysis of triacylglycerol or the esterification of fatty acid (Taylor et al., 1992; Padmini et al., 1994; Hermansyah et al., 2006; Lortie et al., 1993; Reyes and Hill, 1994 ; Xu et al., 2005). Only a small number of kinetic studies on the transesterification of oils by immobilized lipases have been found in the literature (Cheirsilp et al., 2007; Al-Zuhair, 2005; Al-Zuhair et al., 2007). Also the clarification of the mechanisms and the corresponding rate expressions of the transesterification reactions have not been conducted. To elucidate the mechanism of transesterification of two substrates, namely triacylglycerol and alcohol, computer simulation could be a useful tool to independently assess the effects of hypothetical changes in the concentrations of each substrate. Furthermore, it is important to emphasize the effect of alcohol concentration on the mechanism of transesterification since it was reported that alcohol had an inhibitory effect on fatty acid alkyl ester production (Shimada et al., 1999; Chen and Wu, 2003). Although methanol is easily available as an absolute alcohol, it is also involved in enzyme inactivation processes (Meher et al., 2006).

1.2 Objectives of This Research

The purpose of our research was to employ enzymatic method in industrial production of biodiesel from palm oil.

Firstly, the synthesis of fatty acid ethyl esters (FAEE) from palm oil by various lipases in solvent-free system was investigated. For industrial application, immobilization of lipase was optimized. Optimal reaction parameters for synthesis of fatty acid alkyl esters from palm oil were also determined: enzyme loading, water addition, temperature, molar ratio of substrates and three-step addition of alcohols.

Secondly, the kinetics of transesterification of palm oil and ethanol for fatty acid ethyl ester production was investigated. A simple model based on Ping-Pong Bi Bi was proposed to describe the kinetics of the transesterification and hydrolysis reactions. Then, the mathematical models for the reactions, taking into account the effect of ethanol concentration, were considered. In addition, the effects of ethanol concentrations on the production of fatty acid ethyl ester were incorporated into the model.

Thirdly, the feasibility study of using crude and wasted palm oil in biodiesel production by immobilized Lipase was also conducted compared to that of refined palm oil.

Finally, the continuous production of biodiesel in a packed-bed reactor was also studied.

1.3 Literature Review

Palm Olein

Palm oil gives a more valuable olein (an excellent frying oil) and a less valuable stearin (used in part as a replacement for tallow in the oleochemical industry). The oils and their fractions can be further modified by blending with other oils, by partial hydrogenation, or by interesterification. Further fractionation of the palm oil fractions yields an intermediate fraction (PMF, palm midfraction) that can be used as a cocoa butter extender (Gunstone, 1997). Palm oil is characterized by high levels of carotene and of tocopherols and tocotrienols (vitamin E), which can also be isolated or concentrated as products of considerable value. Palm oil fatty acid distillate (PFAD), a by-product of the principal refining procedure, is an important component of animal feed (Gunstone, 1997).

Palm oil is widely used as a food oil with limited non-food uses also. It differs from other commercially available oils in its fatty acid and triacylglycerol (TAG) composition. It contains almost equal amounts of saturated acids (mainly palmitic with some stearic) and unsaturated acids (mainly oleic with some linoleic acid). The proportion of palmitic and oleic acids in the major TAG leads to stability of the crystals. These are very desirable in the production of margarines and shortenings, especially those with high levels of unsaturated acids.

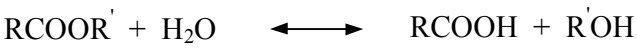
Lipases

Lipases or glycerol ester hydrolases (EC 3.1.1.3) were originally employed for the hydrolysis of ester bonds of TAG to produce free fatty acid (FFA), glycerol (G) and partial acylglycerols (monoacylglycerol, MAG and diacylglycerol, DAG). Lipases occur widely in animals, plants and microorganisms. Numerous mammalian tissues, organs and fluids, such as pancreas, kidney, adipose tissue, heart, brain, muscle and serum, have been known to contain lipases. Among animal lipases pancreatic lipase has been studied most extensively. The hog pancreatic lipase has been studied most extensively, presumably because of its high concentration (2.5% of the total protein in the pig pancreatic juice) and high turnover number. During recent

years considerable attention has been devoted to lipases produced by microorganisms, presumably because of their stability and their practical medical and industrial applications. Several microorganisms produce intracellular or extracellular lipases. Especially, extracellular microbial lipases have high potential for application and are appropriated for mass production. A variety of microorganisims produce lipases. These include the genera of *Candida* yeast; *Rhizopus, Penicillium*, *Aspergillus*, *Geotrichium* and *Mucor* molds; and *Pseudomonas*, *Achromobacter*, and *Staphylococcus* bacteria (Godtfredsen, 1993).

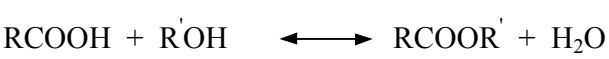
Lipases catalyze three types of reaction. The catalytic action of lipases is reversible. It catalyzes ester synthesis in a microaqeous system. However, in view of biotransformation of oleochemical industry yielding value-added products, the transesterification action seems more worthwhile than hydrolysis and ester synthesis. The difference in free energy involved in TAG hydrolysis is quite small and the net free energy of transesterification is zero. Gandhi (1997) suggested that lipase catalyzed reaction has classically been divided into two main categories: (i) hydrolysis, and (ii) synthesis. Reactions under synthesis category can be further separated: (a) esterification, (b) interesterification, (c) alcoholysis and (d) acidolysis (Figure 1-1).

(i) Hydrolysis

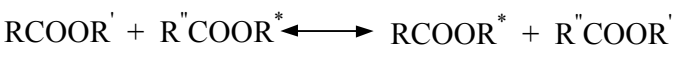


(ii) Synthesis

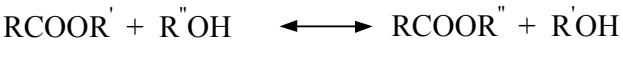
(a) Esterification



(b) Interesterification



(c) Alcoholysis



(d) Acidolysis

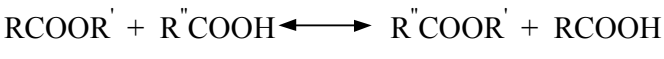


Figure 1-1 Types of reaction catalyzed by lipases.
Source : Gandhi (1997)

Lipases obtained from natural sources can be positionally nonspecific or display one of two kinds of positional specificity: *sn*-1,3 specific or *sn*-2 specific. Non-specific lipases hydrolyse all three ester bonds of triglycerides equally well. Nonspecificity has been observed for lipases from *Chromobacterium viscosum*, *Pseudomonas fluorescens*, *Candida cylindracea*, *Geotrichum candidum*, and *Penicillium cyclopium*, and also for hepatic lipase. Specificity of the *sn*-1,3 type is associated with the preferential release of fatty acid residues from the terminal positions of the glycerol backbone rather than from the central carbon atom, whereas *sn*-2 specificity refers to preferential release from the central carbon atom. The *sn*-1,3 type of specificity has been observed for pancreatic and adipose tissue lipases and lipase from microorganism such as *Rhizopus arrhizus*, *Aspergillus niger*, *Rhizopus delemar* and *Mucor miehei*. The *sn*-2 specificity is extremely rare, and it has been ascribed to a lipase from *Geotrichum candidum* which has a particular ability to hydrolyse oleic and linoleic acids from the *sn*-2 location. A more general classification states that the positional specificity of lipases is not divided clearly into

the above categories; instead it changes continuously from highly specific an-1,3 activity to a very weakly specific or completely nonspecific activity (Malcata *et al*.,1992).

Lipase can be employed in the production of pharmaceuticals, cosmetics, leather, detergents, foods, perfumery, medical diagnostics, and other organic synthetic materials (Gandhi, 1997). Moreover, lipases have found applications in various fields of biotransformations. These can be classified according to the nature of the substrates into three main categories: (i) modification of fats and oils, (ii) acylation/deacylation of carbohydrates and protecting/deprotecting of peptides and (iii) synthesis of chiral compounds. The major focus will be given to the first field. These can be classified depending on the targeted product into the synthesis of MAG, an important class of emulsifiers and the synthesis of structured triglycerides, which are used as, e.g., cocoa-butter equivalents or in nutrition. Furthermore, lipases have found some special applications such as in the selective enrichment of specific fatty acids.

Immobilized lipases

Lipases are normally used in an immobilized form in industry because reuse or continuous use of the immobilized lipase is made possible and the separation of the product from the enzyme is easy. The stability of lipase is often increased by immobilization. The advantages of the various types of available enzyme reactors can also be more readily exploited by using immobilized lipases, especially the use of packed-bed reactors. Brady *et al*. (1988) searched for adsorbents suitable as supports for lipases. Adsorbents, such as celite, cellulose, ethyl cellulose, silica gel, kieselguhr, clay, alumina, CPG-100, carbon, Accurel, Celgard 2500, Profax PP, Microthene HDPE, etc., were screened as possible immobilization supports. Most of hydrophilic materials were found to decrease tremendously the lipase activity upon immobilization. On the other hand, hydrophobic microporous materials such as Accurel and Celgard 2500 were found to provide better performances (Brady *et al*., 1988).

Transesterification by immobilized lipases

Recently, utilization of lipase in the production of fatty acid alkyl esters has been shown to be effective. In the transesterification of soybean oil, 80-90% conversion of esters was obtained using *Rhizopus oryzae* lipase solution (Kaieda *et al.*, 1999). Lipase-catalyzed synthesis reaction selectivity is high and lipase can be immobilized in the support material, which will allow for its recovery and reuse.

Transesterifications of various plant oils using immobilizied lipases have been studied. Dossat *et al.* (2002) and Soumanou and Bornscheuer (2003) both synthesized methyl esters from sunflower oil in solvent-free system using immobilized *Rhizomucor miehei* with conversion of 60% and 80%, respectively. Du *et al.* (2004) synthesized methyl esters from soybean oil using immobilized *Candida antarctica* lipase B with conversion higher than 80% by stepwise additions of methanol. Noureddini *et al.* (2005) synthesized 67% of methyl ester and 65% of ethyl ester from soybean oil using immobilized *Pseudomonas cepacia* lipase. Li *et al.* (2006) reported transesterification of rapeseed oil using combination of immobilizied *Thermomyces lanuginose* lipase and *Candida antarctica* lipase with 95% conversion of esters. Shimada *et al.* (1999) and Watanabe *et al.* (2000) used immobilized *Candida antarctica* lipase (Novozym 435) for the conversion of vegetable oil to biodiesel. Results showed incomplete methanolysis of vegetable oil which was attributed to the inactivation of the enzyme. Stepwise addition of methanol prevented this inactivation and conversions in excess of 98% were obtained.

Several researchers have investigated biodiesel production from palm oil using immobilized lipases. Abigor *et al.* (2000) studied the lipase-catalyzed transesterification of palm kernel oil with different alcohols using lipase PS-30. Talukder *et al.* (2006) used *Candida antarctica* lipase to produce methyl esters from palm oil in presence and absence of organic solvent. However, the bottleneck of lipase-catalyzed biodiesel production is the high price of the enzyme. Since the price of *Candida antarctica* lipase is relatively expensive and far from practical, the study of other low-cost lipases for biodiesel production from palm oil is needed. Kaieda *et al.* (2001) reported that lipases from a number of microorganisms are able to catalyze methanolysis with appropriate water and methanol contents in the reaction mixture. Lipase that is relatively resistant to methanol, such as that from *Pseudomonas cepacia,* will be advantageous in low water content, which is desirable to attain a high reaction rate of transesterification.

Kinetics of reaction catalysed by immobilized lipases

For a simple enzymatic reaction in soluble, the maximum intrinsic rate of reaction is limited by the rate at which lipase and substrate come together in the proper orientation. For the case of hydrolysis reaction, the substrate is often part of the disperse phase of an emulsion, a micelle, or a monolayer which contacts water. These structures may be orders of magnitude larger in size than the supported enzyme for

the case where the carrier exists in powdered form. Thus, the maximum attainable rate is limited by the amount of lipase which can interact with the substrate continuum. In the case of lipases immobilized on continuous supports, or on discrete supports larger in size than the individual droplets of substrate, the above reasoning remains valid provided that the spacing between neighboring molecules of immobilized lipase is still larger than the area of contact between the droplet and the lipase carrier. For this situation, in contrast to the classic Michaelis-Menten rate expression where the reaction rate increases linearly with the total enzyme concentration, a limiting rate is approached as the lipase concentration is increased. In the present case a balance on the total number of adsorption sites is more relevant than a balance on the total number of active sites. This approach leads one to the following rate expression:

$$\frac{-dC_S}{dt} = \frac{V_{max}\ C_E}{K_m + C_E}$$

where the constants are defined as $V_{max} = k_{cat}C_{S(tot)}$ and $K_m = k_{des} / k_{ads}$, and where the physical interpretations of the constants are as follows: V_{max} is the rate when the adsorption sites on the surface of the fat globule are saturated with lipase, and K_m is a pseudo-Michaelis-Menten constant for the above rate expression. When a lipase from *Candida rugosa* was immobilized by adsorption on cellulose, values for V_{max} and K_m changed from ca. 6.48 to 2.92 mol/min, and from ca. 3.88 to 0.54 mg/mL, respectively. The primary mechanistic distinction between this mechanism and the simple Michaelis-Menten mechanism is that in the present case adsorption of lipase at the fluid solid interface (i.e., contact with the substrate molecules) is independent of catalysis in the interfacial plane. Observed K_m values for lipases may thus reflect the extent of adsorption of the lipase at the lipid/water interface rather than the affinity between enzyme and substrate at the active site (Malcata *et al*., 1992).

CHAPTER 2 Materials and Methods

2.1 Materials

Lipase from *Pseudomonas* sp. (lipase PS), *Pseudomonas fluorescens* (lipase AK), *Candida rugosa* (lipase AY) and *Rhizopus delemar* (lipase D) were gifts from Amano Pharmaceutical Co. Ltd., Nagoya, Japan. The supports were hydrophobic polypropylene powder EP200 (Accurel) was a gift from Akzo Nobel (Obernburg, Germany). Palm olein was purchased from Morakot Industry Co. Ltd., Thailand. All other chemicals were also obtained from commercial sources.

2.2 Immobilization

The supports in powdered form (0.2 g) was treated with ethanol before added to 20 ml lipase solution and stirred with a magnetic bar at 100 rpm for 1 h. Afterwards, the suspension was filtered through a Buchner funnel. The immobilized enzyme was washed on the filter paper with another 5.0 ml of 0.1M phosphate buffer pH 7.0 and dried in a vacuum desiccator for 12 h. For this immobilization study, the immobilized yield was calculated using the following formula:

$$\text{Immobilized yield (\%)} = \frac{C_0V_0 - C_fV_f}{C_0V_0} \times 100$$

where C_0 is the initial activity of lipase solution (U ml^{-1}); V_0 is the initial volume of lipase solution (ml); C_f is the lipase activity of the filtrate (U ml^{-1}); and V_f is the filtrate volume (ml).

2.3 Transesterification

Each lipase was investigated for its ability to catalyze transesterification of palm oil. The substrate mixture consisted of 5 g palm oil, 3 molar equivalents of ethanol to palm oil and 1 ml 0.1 M phosphate buffer pH 7.0 containing 30 U of enzyme. The reaction was maintained at 45 ◦C.

2.4 Analysis

The course of transesterification was monitored by intermittent sampling (150 mg) followed by chloroform extraction. The extract was analyzed for FAEE, fatty acid methyl esters (FAME), triacylglycerol (TAG), 1,3-diacylglycerol (1,3-DAG), 1,2-diacylglycerol (1,2-DAG), MAG and free fatty acid (FFA) using a thin-layer

chromatography/flame ionization detection (TLC/FID) (IATROSCAN MK5, Iatron Laboratories Inc., Tokyo) (Rosu *et al.*, 1997). In this paper, the percentage of peak area was assumed as percentage content of the corresponding compound. Hydrolytic activity of the lipase was assayed by a modified cupric acetate method (Lee and Rhee, 1993). One unit of hydrolytic activity is defined as the amount of the enzyme that liberates 1μmole equivalent of palmitic acid from palm olein in 1 min at 45°C.

All the data presented in this paper at various conditions were the mean of three experiments. Appropriate tests of significance, analysis of variance (ANOVA) and confident difference at 5% level were used in the data evaluations.

Ordinary differential equations were solved by the Runge-Kutta single-step fourth-order method (Danby, 1997). The programs were coded in the Visual Basic program ver. 6.0 (Microsoft Inc., USA).

2.5 Continuous transesterification

The reactor consisted of a glass cylinder with a working volume of 7.85 ml (inside diameter 1 cm, height 10 cm). The immobilized lipase was packed into the column. The temperature of the reactor was controlled by running water into the jacket at 45°C. The substrate consisted of palm oil and alcohol in the molar ratio of 1:3.

CHAPTER 3 Results and Discussion

3.1 Screening of lipase

In biodiesel production, the preliminary screening of lipases was carried out with randomly chosen commercial available lipases. In a typical reaction, the same amount of 30 U hydrolytic activity of each enzyme was evaluated in transesterification of 5 g palm oil with 3 molar equivalents of ethanol. Four commercial lipases were screened for their ability to produce FAEE from palm olein. The screening results for the tested lipases are presented in Table 3-1. Reaction products are presented as % FAEE content in the reaction mixture.

Table 3-1 FAEE production using commercial lipases

Lipase	TAG (%)	FFA (%)	FAEE (%)
PS	0.68 ± 0.63^{c}	6.69 ± 1.27^{a}	56.6 ± 2.56^{a}
AK	92.5 ± 0.95^{a}	0.34 ± 0.13^{d}	4.22 ± 0.63^{b}
AY	91.0 ± 0.57^{b}	1.98 ± 0.38^{b}	1.74 ± 0.42^{c}
D	93.4 ± 0.23^{a}	0.76 ± 0.12^{c}	1.46 ± 0.16^{c}

Mean ± S.D. (n = 3).

Different letter in each column means statistically significant differences ($p<0.05$).

The formation of free fatty acids is also included in this table since the presence of water in the reaction medium naturally promotes the competing hydrolysis reaction. Among the tested lipases, lipase PS from *Pseudomonas* sp. showed the highest activity toward the transesterification of palm oil with ethanol. Other lipases showed very little activity toward the transesterification reaction. After 6 h of reaction with lipase PS, the product contained 57% FAEE, 6% of fatty acids, 24% of monoglycerides, 13% of diglyceride, and 0% of triglycerides. Therefore, lipase from *Pseudomonas* sp. was the most promising one for the transesterification of palm oil in this study as it was also reported in the transesterification of soybean oil (Kaieda *et al.*, 2001; Noureddini *et al.*, 2005) and *Jatropha* oil (Shah and Gupta, 2007). It is likely that lipase from *Pseudomonas* sp. has much higher methanol resistance than those from the others (Kaieda *et al.*, 2001), this makes it more attractive for use as an enzyme in alcoholysis reaction of palm oil.

3.2 Optimal conditions for enzyme immobilization

By immobilizing the enzyme on support material, it is possible to reuse enzyme and reduce the costs of the enzyme in industrial application. In this study, selected lipase PS was immobilized on Accurel, hydrophobic polypropylene powder. The immobilization mechanism of lipase on Accurel was simple adsorption, whereby the enzyme adheres to the surface of the support particles by Van der Waals forces of attraction (Murray *et al.*, 1997). Many lipases display interfacial activation phenomena in the presence of a hydrophobic surface. The activation has been related to a conformational change of the lipase that exposes to the hydrophobic substrate a wide hydrophobic surface surrounding the catalytic site (Brzozowski *et al.*, 1991). For the same reason, lipases are strongly adsorbed by hydrophobic surfaces. Hence, lipase immobilization by adsorption may not only improve the stability and ease of product separation, but also enzyme activity.

3.2.1 Effect of enzyme concentration

The effect of the enzyme concentration on immobilization of lipase PS with Accurel was investigated as shown in Table 3-2. The activity of the immobilized lipase increased with increasing enzyme concentration. This could be explained that increasing the concentration of enzyme in solution increases the driving force for adsorption. However, at enzyme concentration higher than 50 U ml^{-1} all lipase molecules could not load onto Accurel resulted in low immobilization yield. When immobilized yield was considered, the concentration of enzyme with 50 U ml^{-1}, was suitable for lipase PS immobilization on Accurel.

Table 3-2 Effect of enzyme loading on immobilization of lipase PS with Accurel

Enzyme concentration (U ml^{-1})	Immobilized yield (%)	Immobilized lipase activity (U mg^{-1})
5	46.56 ± 5.04^{d}	0.045 ± 0.015^{d}
30	58.44 ± 1.68^{c}	0.082 ± 0.002^{c}
50	83.79 ± 2.27^{a}	0.258 ± 0.011^{b}
100	71.86 ± 1.51^{b}	0.649 ± 0.003^{a}

Mean ± S.D. (n = 3).

Different letter in each column means statistically significant differences ($p<0.05$).

3.2.2 Effect of immobilization time

The effect of time on immobilization of lipase PS on Accurel was studied. The immobilized lipase activity increased while the immobilized yield decreased when the immobilization time increased (Table 3-3).

Table 3-3 Effect of time on immobilization of lipase PS with Accurel

Immobilization time (min)	Immobilized yield (%)	Immobilized lipase activity (U mg^{-1})
15	91.15 ± 0.72^{a}	0.101 ± 0.003^{d}
30	83.79 ± 2.27^{b}	0.258 ± 0.011^{c}
60	81.24 ± 1.04^{b}	0.285 ± 0.010^{b}
90	73.93 ± 0.80^{c}	0.350 ± 0.002^{a}

Mean ± S.D. (n = 3).

Different letter in each column means statistically significant differences ($p<0.05$).

Since Accurel is a hydrophobic polymer, a longer immobilization time was, more floatation and separation of Accurel from lipase solution occurred and resulting low immobilized yield. The immobilization time of 15 min gave highest immobilized yield but much lower immobilized lipase activity compared to that of immobilization time of 30 min. Thus, the immobilization time of 30 min, which gave high immobilized yield and immobilized activity, was sufficient to immobilize lipase PS on Accurel. According to Montero *et al.* (1993), *Candida rugosa* lipase was also rapidly adsorbed on Accurel and more than 60% of the activity disappeared from enzyme solution after 1 min of incubation.

3.3 Comparison of FAEE production by free and immobilized lipase PS

The immobilized lipase PS produced under the optimal immobilization conditions established in this study was tested for FAEE production and compared with free lipase. Figure 3-1 shows time course of FAEE production by immobilized and free lipase PS.

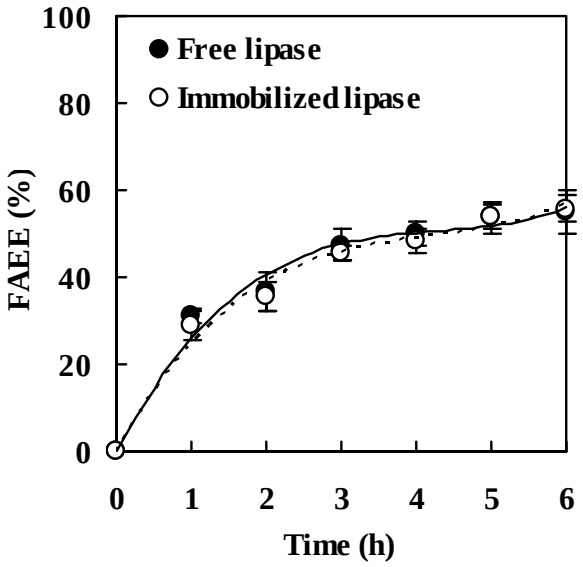


Figure 3-1 Time course of FAEE production by immobilized and free lipase PS. Reaction condition; 5 g of palm oil; molar ratio of palm oil to ethanol of 1:3; 30 U of immobilized and free lipase PS; 1.00 ml of water addition and at 45°C.

There was no significant difference in FAEE production by immobilized and free lipase. Both free and immobilized lipase PS gave a maximum FAEE yield of 60% at 6 h. This result suggests that the lipase is being adsorbed onto Accurel in such a way as not to obscure the active site, resulting in remain of enzyme activity. The result also shows that mass transfer limitation in Accurel porous supports could be neglected as it was reported for other porous supports (Chen and Wang, 1998; Romero *et al.*, 2007). Salis *et al.* (2003) reported that the internal diffusion has seldom been considered in much biocatalysis work in organic media, since the reactions are usually not very fast. Shah and Gupta (2007) indicated that the immobilized *Pseudomonas cepacia* lipase on celite gave higher yield of biodiesel from *Jatropha* oil than free lipase. This is presumed to be because of larger surface area of the biocatalyst preparation in the immobilized form. Therefore, the immobilized enzymes give better catalytic performance in non-aqueous media. However, this property also depends on type of the support. In this study, there was no significant difference in activities of free lipase and immobilized lipase on Accurel.

3.4 Optimization of biodiesel production from palm oil by immobilized lipase PS

3.4.1 Effect of immobilized lipase PS loading

The effect of immobilized lipase PS loading based on oil weight on FAEE production from palm oil was presented in Figure 3-2.

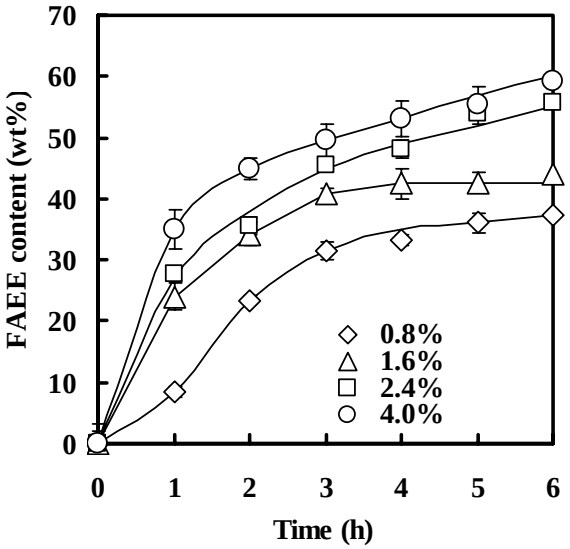


Figure 3-2 Effect of immobilized lipase PS loading on FAEE production from refined palm oil. Reaction condition; 5 g of palm oil; molar ratio of palm oil to ethanol of 1:3; water addition of 20% based on oil weight and at 45°C.

The FAEE yield was enhanced by increasing the amount of immobilized lipase and it was highest at 4%. Therefore, immobilized lipase PS at 4% was used for transesterification of 5 g of palm oil. Similar trends were observed for the transesterification of various oils (Kaieda *et al.*, 1999; Noureddini *et al.*, 2005; Li *et al.*, 2006; Shah and Gupta, 2007).

3.4.2 Effect of free fatty acid

The cost of oil sources accounts for a large part in the biodiesel production, a crude palm oil and a used palm oil sources were also explored for biodiesel production with immobilized lipase. Since an enzymatic method have been sought to produce methyl esters from acid oil (Ghosh and Bhattacharyya, 1995; Tueter et al., 2004; Watanabe et al., 2007), the effect of fatty acid (oleic acid) on biodiesel production by immobilized lipase was investigated (Figure 3-3).

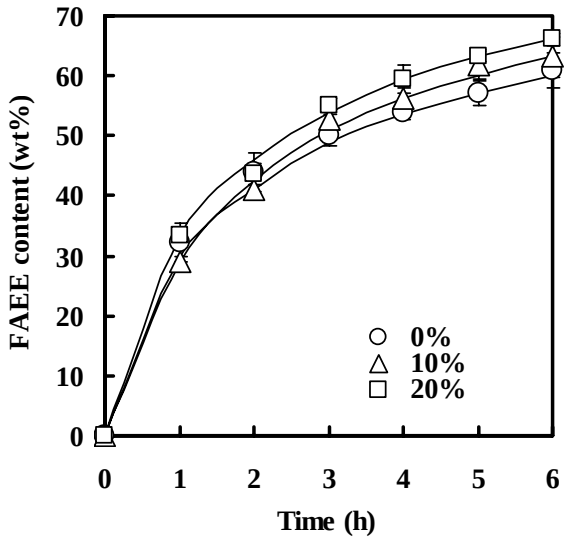


Figure 3-3 Effect of free fatty acid on FAEE production from refined palm oil. Reaction condition; 5 g of palm oil; 4% of immobilized lipase; molar ratio of palm oil to ethanol of 1:3; water addition of 20% based on oil weight and at 45°C.

The fatty acid content was varied from 0% to 20% (by weight of oil). Fatty acid ethyl ester (FAEE) production increased with increasing amounts of fatty acid. It was found that the range of fatty acid of 0-20% have no inhibition on the transesterification reaction. Figure 3-3 indicated that higher initial concentrations lead to a faster initial rate of FAEE production. In addition, the higher initial concentrations result in greater extents of incorporation of fatty acid into oil at equilibrium.

3.4.3 Effect of temperature

The effect of temperature on FAEE production was studied over the temperature range of 30–55°C (Figure 3-4). The temperature effect is related to the enzyme activity and stability. Increasing temperature from 30 to 45°C, FAEE production increased from 52 to 61%. At higher temperature 55 °C, there was no significant further increase in FAEE production compared to the temperature at 45°C. Therefore, the temperature of 45°C was chosen for transesterification of palm oil from the economical point of view. Most of lipase-catalyzed biodiesel productions have also been tested at 45°C (Nelson *et al.*, 1996).

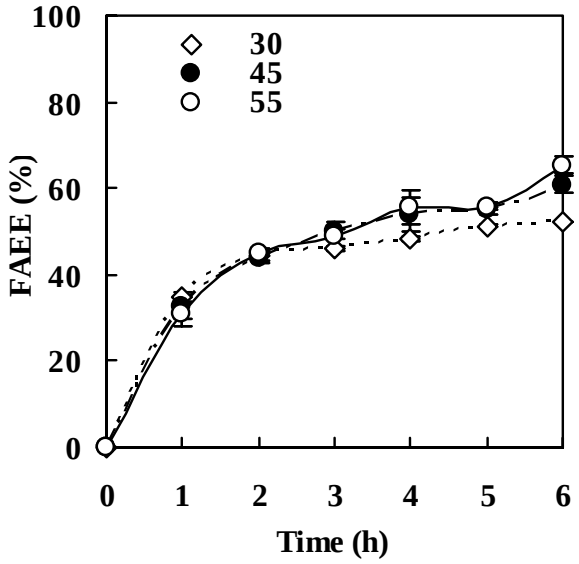


Figure 3-4 Effect of temperature on FAEE production from palm oil. Reaction condition; 5 g of palm oil; 4% of immobilized lipase; molar ratio of palm oil to ethanol of 1:3; water addition of 20% based on oil weight and at 45°C.

3.4.4 Comparison between methanol and ethanol as alcohol substrate

Experiments were performed to optimize the amount of fatty acid alkyl ester production by varying the alcohol concentration. Optimum alcohol requirements were determined for both ethanol and methanol as shown in Figure 3-5. The amount of alcohol added was varied from 1 to 3 molar equivalents based on the moles of palm oil. An increase in the number of moles of alcohol with respect to the palm oil resulted in an increase in the production rates and yields of FAEE and FAME as shown in Figure 3-5. The optimum alcohol concentration was determined at 3 molar ratio of alcohol to palm oil for ethanol and methanol where about 61% of FAEE and 60% of FAME were formed, respectively at 6 h. At the same molar ratio of alcohol, the production rate and yield of FAME were higher than that of FAEE. Similar results were observed by Salis *et al.* (2005).

Although methanol is easily available as an absolute alcohol, it is involved in enzyme inactivation processes (Meher *et al.*, 2006). The formation of FAEE is environmentally attractive because unlike methanol, ethanol is produced from renewable resources. Also, ethanol has better solvent properties than methanol for solubility of oil (Shimada *et al.*, 2002). When 2 or 3 molar equivalents of alcohols were added to the reaction mixture, the yields of esters were 52 and 60% for FAEE

and 54 and 61% for FAME, although theoretically it should have reached 66 and 100%. This result indicates that the lipase was probably inactivated when the alcohol content is high. Chen and Wu (2003) reported that when the lower alcohol in the mixture was high, it caused deactivation of the enzyme due to the immiscibility between triglycerides and alcohol.

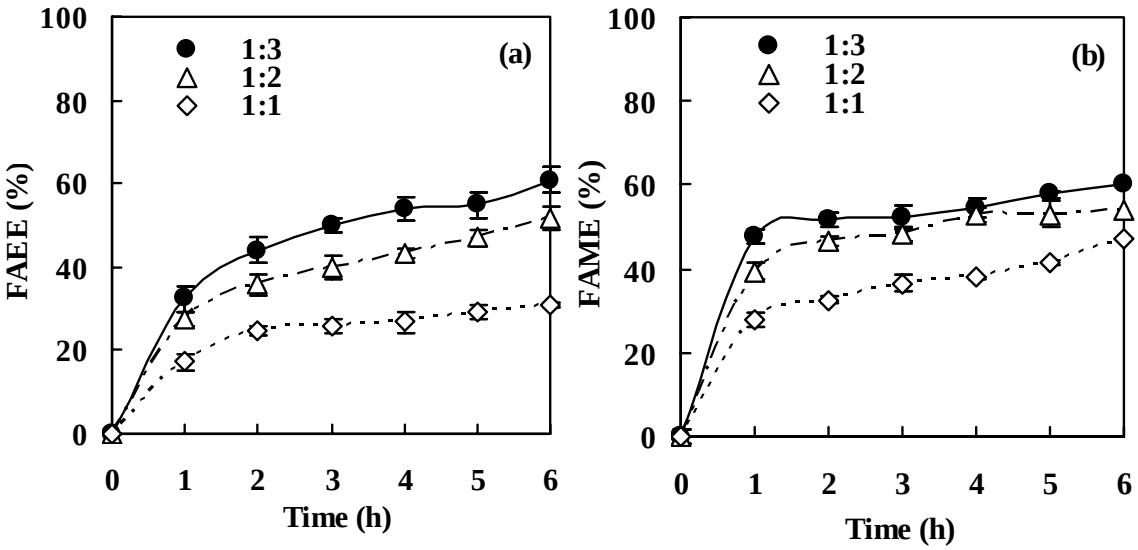


Figure 3-5 Effect of molar ratio of ethanol on FAEE production (a) and molar ratio of methanol on FAME production (b). Reaction condition; 5 g of palm oil; 4% of immobilized lipase PS; 20% of water addition at 45°C.

Therefore, to avoid lipase inactivation by ethanol and methanol a method of adding stepwise was applied. The results are shown in Figure 3-6. When alcohol was added to the reaction mixture, the FAEE and FAME yields increased. The FAEE and FAME contents reached to 76 and 75%, respectively at 5 h with stepwise additions of alcohols. It is remarkable that the stepwise addition of alcohol exhibits the highest conversions of palm oil to biodiesel. Since alcohols were maintained at low concentration in the reaction mixture during stepwise addition of alcohols, the effect of deactivation by alcohols was softened and the reusability of immobilized lipase PS could be improved.

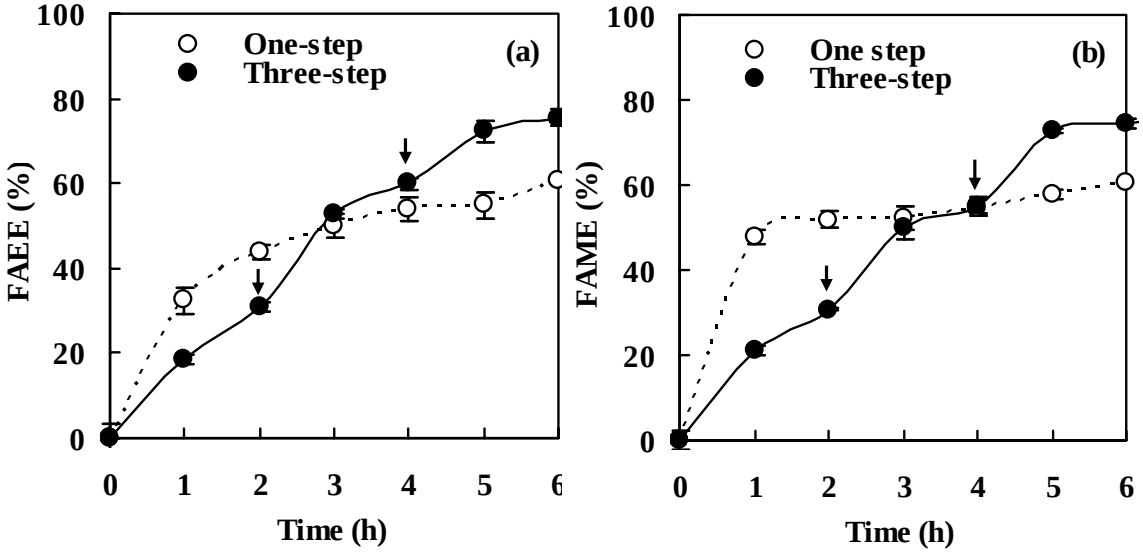


Figure 3-6 Effect of three-step addition of ethanol on FAEE production (a) and methanol on FAME production (b) at 0, 2 and 4 h as arrows indicating. Reaction condition; 5 g of palm oil; 4% of immobilized lipase PS; 20% of water addition; total 3 molar equivalents of alcohol; and at 45°C.

3.4.5 Effect of water addition

The important factor that affects the activity of lipase in microaqueous reaction system is the amount of water in the reaction system. Most of enzymes require a certain amount of water to maintain their active conformation (Piyatheerawong et al., 2004). In many cases, the reaction rate is low at very low water content, and increases when more water is present. Since, lipase activity generally depends on the available interfacial area. With the increased addition of water, the amount of water available for oil to form oil–water droplets increases, thereby, increasing the available interfacial area. However, since lipases usually catalyze hydrolysis in aqueous media, excess water may also stimulate the competing hydrolysis reaction. The optimum water content is a compromise between minimizing hydrolysis and maximizing enzyme activity for the transesterification reaction (Noureddini *et al.*, 2005). The water content was also reported that it could prevent the inactivation of lipase by alcohol (Kaida et al., 2001). Since the composition of oil in refined, crude and used palm oil are different as shown in Table 3-4 especially water content, the effect of water addition on FAEE production from each oil were determined as shown in Figure 3-7.

Table 3-4 Composition of oils

Component (%)	Refined palm oil	Crude palm oil	Used palm oil
TAG	98.34	85.93	93.82
DAG	1.66	2.28	4.90
MAG	-[a]	2.58	0.18
FFA	-[a]	9.21	1.1
Water content (%)	0.36	0.11	0.85

[a] – could not detect

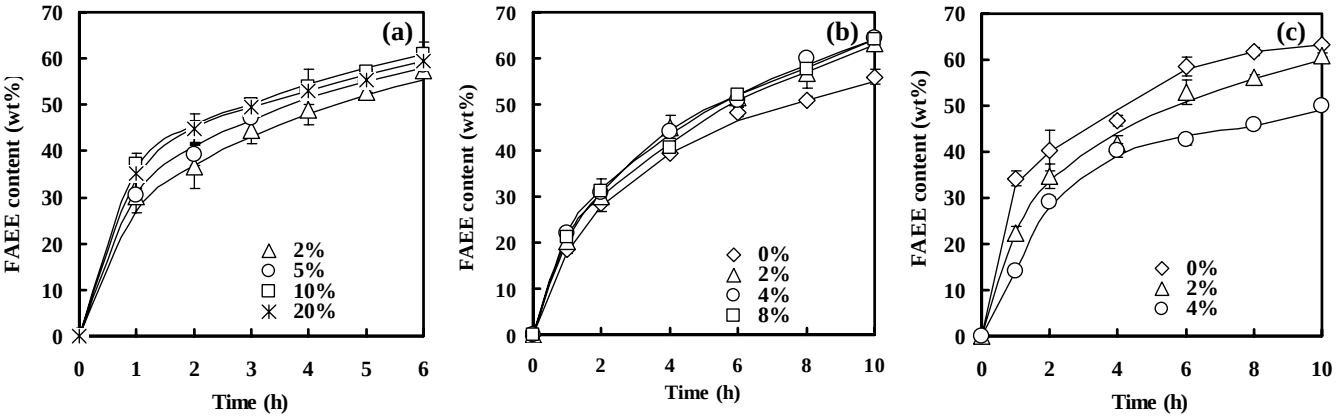


Figure 3-7 Effect of water addition on FAEE production from refined palm oil (a), crude palm oil (b) and used palm oil (c). Reaction condition; 5 g of palm oil; 4% of immobilized lipase; molar ratio of palm oil to ethanol of 1:3 at 45°C.

The results show that increasing water addition led in higher conversion rate in refined and crude palm oil. However, there was no significant difference on FAEE production profile at the water addition beyond 10% in refined palm oil and 2% in crude palm oil. In contrast, when the water addition in used palm oil increases the FAEE synthesis rate decreases and the yield get lower. It was also found that the effect of water addition in refined and crude palm oil has less pronounced on FAEE yield compared to that of used palm oil. This might be due to high water content in used palm oil (Table 3-4) could reduce the equilibrium conversion for reversible reactions. Based on the above results, the transesterification of refined, crude and used palm oil were conducted with water addition of 10%, 2% and 0%, respectively.

3.4.6 Effect of molar ratio of ethanol on refined, crude and used palm oil

Experiments were performed to optimize the amount of FAEE production for refined, crude and used palm oil by varying the ethanol concentration. The amount of ethanol added was varied from 1 to 3 molar equivalents based on the moles of palm oil. An increase in the number of moles of ethanol with respect to the palm oil resulted in an increase in the production rate and yield of FAEE as shown in Figure 3-8. The optimum ethanol concentration was determined at 3 molar ratio of ethanol to palm oil for refined, crude and used palm oil where about 97%, 94% and 96% of FAEE were formed, respectively at 48 h (Table 3-5). The initial production rates and yields of FAEE for refined, crude and used palm oil were almost the same at each molar ration of ethanol to palm oil. This result indicates that the different compositions in refined, crude and used palm oil did not affect the FAEE production at optimal water content for each. This phenomenon was contrast to the previous report that the reaction rate of crude soybean oil was much lower than that with the refined one due to the inhibitive effect on the enzymatic activity caused by lipids existing in crude oil sources (Du et al., 2004).

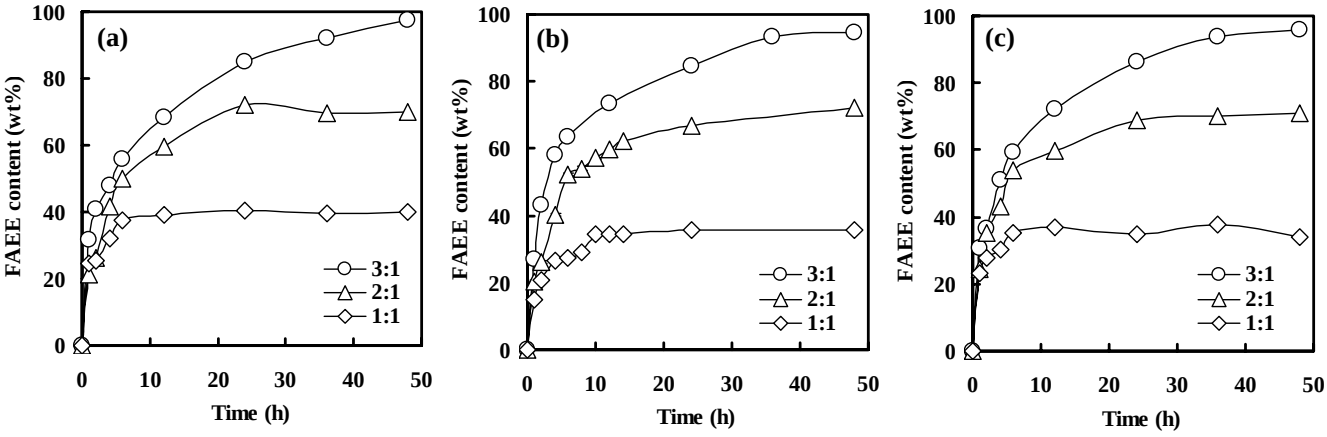


Figure 3-8 Effect of molar ratio of ethanol on FAEE production from refined palm oil (a), crude palm oil (b) and used palm oil (c). Reaction condition; 5 g of palm oil; 4% of immobilized lipase; molar ratio of palm oil to ethanol of 1:3 at 45°C.

To avoid lipase inactivation by ethanol a method of adding stepwise was applied to each type of oils. The results are shown in Figure 3-9. When 3 molar ratio of ethanol was stepwise added to the reaction mixture at 0, 2 and 4 h, the production rate of FAEE increased and the FAEE contents reached to 94% for crude palm oil and 95% for refined and used palm oil within 24 h (Table 3-5) and reached equilibrium

conversion of 99%, 98% and 96% at 48 h for refined, crude and used palm oil, respectively. It is remarkable that the stepwise addition of ethanol could reduce the inhibition and higher conversion rate of palm oil to biodiesel was obtained compared to one-step addition. Since ethanol was maintained at low concentration in the reaction mixture during stepwise addition, the effect of deactivation by ethanol was softened and the reusability of immobilized lipase PS could be improved.

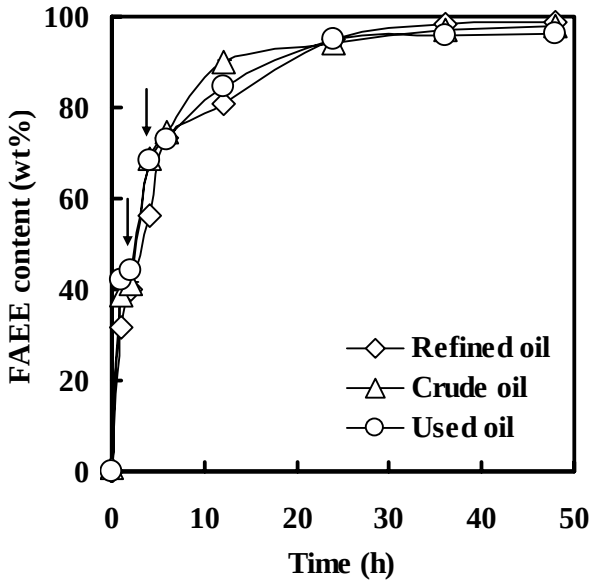


Figure 3-9 Effect of three-step addition of ethanol on FAEE production (a) and methanol on FAME production (b) at 0, 2 and 4 h as arrows indicating. Reaction condition; 5 g of palm oil; 50 U of immobilized lipase PS; 0.50 ml of water addition; total 3 molar equivalents of alcohol; and at 45°C.

Table 3-5 Conversion achieved for refined, crude and used palm oil with stepwise addition of ethanol compared to one-step addition

Different oil	One-step addition (%)			Stepwise addition (%)		
	12 h	24 h	48 h	12 h	24 h	48 h
Refine palm oil	69	85	97	81	95	99
Crude palm oil	73	85	94	90	94	98
Used palm oil	72	86	96	85	95	96

3.5 Modeling of lipase catalytic reaction

To elucidate the mechanism of transesterification of two substrates, namely triacylglycerol and alcohol, computer simulation could be a useful tool to independently assess the effects of hypothetical changes in the concentrations of each substrate. Furthermore, it is important to emphasize the effect of alcohol concentration on the mechanism of transesterification since it was reported that alcohol had an inhibitory effect on fatty acid alkyl ester production (Shimada et al., 1999; Chen and Wu, 2003). Although methanol is easily available as an absolute alcohol, it is also involved in enzyme inactivation processes (Meher et al., 2006). Ethanol is environmentally attractive because unlike methanol, ethanol could be produced from renewable resources and used in biodiesel production (Yamada et al., 2007). Also, ethanol is a better solvent than methanol for oil (Shimada et al., 2002). In this study, palm oil and ethanol were used as reactants. The kinetics of transesterification of palm oil and ethanol for fatty acid ethyl ester production was investigated. First, a simple model based on Ping-Pong Bi Bi was proposed to describe the kinetics of the transesterification and hydrolysis reactions. Then, the mathematical models for the reactions, taking into account the effect of ethanol concentration, were considered. Unlike previous reports in the literature, the present models are able to account for the effects of the concentrations of all chemical species participating in the transesterification reaction. In addition, the effects of ethanol concentrations on the production of fatty acid ethyl ester were incorporated into the model.

3.5.1 Modeling of the lipase catalyzed transesterification reaction

Mechanisms of lipase catalyzed transesterification reaction based on hydrolysis and esterification of triacylglycerol, diacylglycerol, monoacylglycerol, free fatty acid and ethanol were considered. The starting point for the development of an appropriate generic mechanism describing the transesterification reactions is the mechanism of the ester bond hydrolysis reaction to produce free fatty acid, which constitutes the first step. This ester hydrolysis step is followed by the esterification step that produces a new ester bond by reacting the newly created free fatty acid with the incoming alcohol group. Figure 3-10 shows the hydrolysis and esterification steps with the free enzyme (E) reacting with triacylglycerol (T) to form the first complex (E·T) and then T is hydrolyzed to diacylglycerol (D) and fatty acid (F).

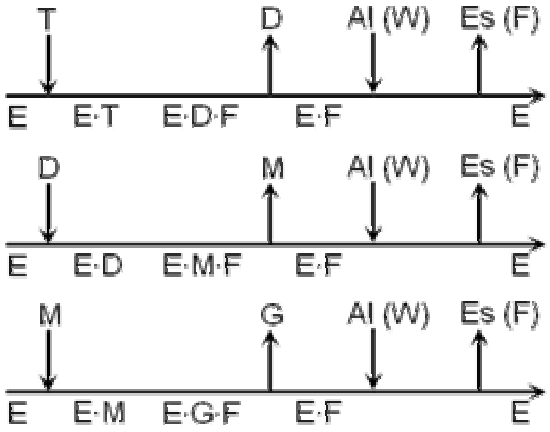


Figure 3-10 Schematic diagram of Ping Pong Bi Bi mechanisms for stepwise transesterification of palm oil.

Subsequently, D is released from the second complex (E·D·F) to form the third complex (E·F). This complex might react with alcohol (Al) through an alcoholysis reaction to form an alkyl ester (Es) or with water (W) through a hydrolysis reaction to form free F. The mechanisms for the hydrolysis of D and monoacylglycerol (M) are also similar to that described above. However, the precise point in the kinetic cycle at which the ethanol molecule enters the active site is unknown, therefore three mechanisms were proposed. The models differ from one another with respect to the rate-limiting step and the point at which the ethanol molecule enters the catalytic cycle. Rate expressions corresponding to these mechanisms are developed and tested for consistency with the experimental results with various ethanol concentrations. Three mechanisms share three basic assumptions: (1) since preliminary experimental data indicated that the reaction rate is sufficiently slow so that the mass transfer limitations imposed by the porous support are negligible. This has been also reported in the literature (Reyes and Hill, 1994; Chen and Wang, 1998; Romero et al., 2007), the possible mass transfer limitations in this reaction system were ignored; (2) all the fatty acids released from the triacylglycerol substrate may be lumped together and treated as a single constituent (F); (3) the inhibition of enzyme activity by alcohol follows a competitive inhibition mechanism. Then, three proposed kinetic models and numerical determination of parameters were applied for simulating the effect of the ethanol concentration on the kinetic reactions of transesterification of palm oil by the immobilized lipase.

Mechanism 1

In mechanism 1, starting with the reaction network shown in Figure 3-11, one can derive many rate expressions for the various cases in which different steps are taken as rate determining. All reactions for ester production were classed into two groups: one was hydrolysis and another was esterification. Any hydrolysis reaction that produced free fatty acid included a stepwise process of hydrolysis of triacylglycerol, diacylglycerol and monoacylglycerol. The esterification reaction for fatty acid ethyl ester with excess ethanol is a synthesis reaction. The transesterification reaction therefore involves sequential execution of the hydrolysis and esterification steps, and thus requires multiple entrances and exits of reactant and product species in such a manner as to render the overall mechanism as the Ping-Pong type. Alternatively, some lipases have been reported to exhibit a rate-determining step at the point where the enzyme breaks the complex of enzyme and substrate (Reyes and Hill, 1994). In this mechanism, the expressions for the rates of appearance of hydrolyzed intermediates and synthesized fatty acid ethyl ester are assumed to be rate-limiting steps and the remaining steps are assumed to be in rapid equilibrium.

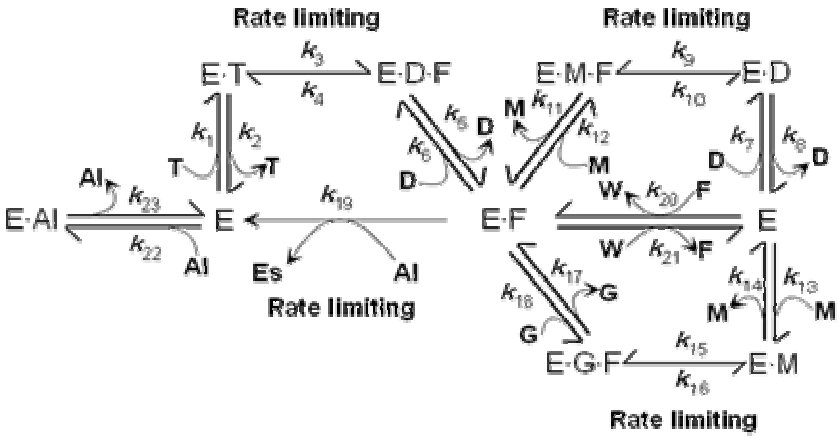


Figure 3-11 Conceptual scheme of overall reaction mechanism 1.

The appearance rates of hydrolyzed intermediates including triacylglycerol (*T*), diacylglycerol (*D*), monoacylglycerol (*M*) and glycerol (*G*) are expressed as in Eqs. (1-4). The appearance rates of free fatty acid (*F*), water (*W*), fatty acid ethyl ester (*Es*) and ethanol (*Al*) from the hydrolysis and esterification steps are presented in Eqs. (5-7).

$$\frac{d[T]}{dt} = -k_3[E \cdot T] + k_4[E \cdot D \cdot F] \tag{1}$$

$$\frac{d[D]}{dt} = k_3[E \cdot T] - k_4[E \cdot D \cdot F] - k_9[E \cdot D] + k_{10}[E \cdot M \cdot F] \tag{2}$$

$$\frac{d[M]}{dt} = k_9[E \cdot D] - k_{10}[E \cdot M \cdot F] - k_{15}[E \cdot M] + k_{16}[E \cdot G \cdot F] \tag{3}$$

$$\frac{d[G]}{dt} = k_{15}[E \cdot M] - k_{16}[E \cdot G \cdot F] \tag{4}$$

$$\frac{d[F]}{dt} = -\frac{d[W]}{dt} = k_3[E \cdot T] - k_4[E \cdot D \cdot F] + k_9[E \cdot D] - k_{10}[E \cdot M \cdot F] + k_{15}[E \cdot M] - k_{16}[E \cdot G \cdot F] - k_{19}[E \cdot F] \tag{5}$$

$$\frac{d[Es]}{dt} = k_{19}[E \cdot F][Al] \tag{6}$$

$$\frac{d[Al]}{dt} = -k_{22}[E][Al] + k_{23}[E \cdot Al] - k_{19}[E \cdot F][Al] \tag{7}$$

where E is the free enzyme concentration and $E{\cdot}T$, $E{\cdot}D$, $E{\cdot}M$, $E{\cdot}F$, $E{\cdot}Al$, $E{\cdot}D{\cdot}F$, $E{\cdot}M{\cdot}F$ and $E{\cdot}G{\cdot}F$ are different complexes between the enzyme and the species defined above. The concentrations of the different enzymatic complexes can be expressed in terms of the free enzyme concentration by means of the following pseudoequilibrium relationships:

$$[E \cdot T] = \frac{k_1}{k_2}[E][T] \tag{8}$$

$$[E \cdot D] = \frac{k_7}{k_8}[E][D] \tag{9}$$

$$[E \cdot M] = \frac{k_{13}}{k_{14}}[E][M] \tag{10}$$

$$[E \cdot F] = \frac{k_{20}}{k_{21}}\frac{[E][F]}{[W]} \tag{11}$$

$$[E \cdot Al] = \frac{k_{22}}{k_{23}}[E][Al] \tag{12}$$

$$[E \cdot D \cdot F] = \frac{k_6}{k_5}[E \cdot F][D] = \frac{k_6 k_{20}}{k_5 k_{21}}\frac{[E][F]}{[W]}[D] \tag{13}$$

$$[E \cdot M \cdot F] = \frac{k_{12}}{k_{11}}[E \cdot F][M] = \frac{k_{12} k_{20}}{k_{11} k_{21}}\frac{[E][F]}{[W]}[M] \tag{14}$$

$$[E \cdot G \cdot F] = \frac{k_{18}}{k_{17}}[E \cdot F][G] = \frac{k_{18}k_{20}}{k_{17}k_{21}}\frac{[E][F]}{[W]}[G] \quad (15)$$

The total enzyme concentration (E_T) is given by

$$E_T = E + E \cdot T + E \cdot D + E \cdot M + E \cdot F + E \cdot D \cdot F + E \cdot M \cdot F + E \cdot G \cdot F + E \cdot Al \quad (16)$$

By substitution of Eqs. (8-16) into Eqs. (1-7), and algebraic manipulation of the resulting equations, the following rate expressions were obtained:

$$\frac{d[T]}{dt} = (-V_{mT}[T] + V_{rT}\frac{[F][D]}{[W]})[E^*] \quad (17)$$

$$\frac{d[D]}{dt} = (V_{mT}[T] - V_{rT}\frac{[F][D]}{[W]} - V_{mD}[D] + V_{rD}\frac{[F][M]}{[W]})[E^*] \quad (18)$$

$$\frac{d[M]}{dt} = (V_{mD}[D] - V_{rD}\frac{[F][M]}{[W]} - V_{mM}[M] + V_{rM}\frac{[F][G]}{[W]})[E^*] \quad (19)$$

$$\frac{d[G]}{dt} = (V_{mM}[M] - V_{rM}\frac{[F][G]}{[W]})[E^*] \quad (20)$$

$$\frac{d[F]}{dt} = -\frac{d[W]}{dt} = (V_{mT}[T] + V_{mD}[D] + V_{mM}[M] - \frac{[F]}{[W]}(V_{rT}[D] + V_{rD}[M] + V_{rM}[G] + V_{eEs}[Al]))[E^*]$$

(21) $$\frac{d[Es]}{dt} = -\frac{d[Al]}{dt} = (V_{eEs}\frac{[F]}{[W]}[Al])[E^*] \quad (22).$$

The $[E^*]$ in Eqs. (17-22) is

$$[E^*] = \frac{[E_T]}{1 + K_{mT}[T] + K_{mD}[D] + K_{mM}[M] + K_{mF}\frac{[F]}{[W]}(1 + K_{mDF}[D] + K_{mMF}[M] + K_{mGF}[G]) + \frac{Al}{K_I}} \quad (23)$$

where V_{mT}, V_{mD} and V_{mM} are rate constants for hydrolysis of *T*, *D* and *M*, respectively. V_{eEs} is the rate constant for esterification of fatty acid ethyl ester. These are defined as:

$$V_{mT} = \frac{k_3 k_1}{k_2},\ V_{mD} = \frac{k_9 k_7}{k_8},\ V_{mM} = \frac{k_{15}k_{13}}{k_{14}} \text{ and } V_{eEs} = \frac{k_{19}k_{20}}{k_{21}} \ .$$

V_{rT}, V_{rD} and V_{rM} are rate constants for re-esterification of *D*, *M* and *G*, respectively and defined as:

$$V_{rT} = \frac{k_4 k_6 k_{20}}{k_5 k_{21}},\ V_{rD} = \frac{k_{10}k_{12}k_{20}}{k_{11}k_{21}} \text{ and } V_{rM} = \frac{k_{16}k_{18}k_{20}}{k_{17}k_{21}} \ .$$

K_{mT}, K_{mD}, K_{mM}, K_{mF}, K_{mDF}, K_{mMF} and K_{mGF} are equilibrium constants for *T*, *D*, *M*, *F*, *D*·*F*, *M*·*F* and *G*·*F* , respectively and defined as:

$$K_{mT} = \frac{k_1}{k_2},\ K_{mD} = \frac{k_7}{k_8},\ K_{mM} = \frac{k_{13}}{k_{14}},\ K_{mF} = \frac{k_{20}}{k_{21}},\ K_{mDF} = \frac{k_6 k_{20}}{k_5 k_{21}},\ K_{mMF} = \frac{k_{12}k_{20}}{k_{11}k_{21}} \text{ and } K_{mGF} = \frac{k_{18}k_{20}}{k_{17}k_{21}}$$

.

K_I is the inhibition constant for ethanol inhibition which is defined as:

$$K_I = \frac{k_{23}}{k_{22}}.$$

Eventually, in this mechanism there are 15 unknown parameters. These parameters were estimated by fitting the model equations with the experimental data obtained with various ethanol concentrations.

Mechanism 2

Mechanism 2 differs from mechanism 1 with respect to the step involving the decomplexation of the enzyme and intermediates after the hydrolysis reaction. It is generally accepted that when the substrate is bound to the active site, the crucial catalytic step is the hydrolysis of the ester bond and forms a mixed acyl complex of liberated fatty acid and enzyme. Once this mixed acyl complex is formed, the hydrolyzed intermediate is released (Malcata et al., 1992). Accordingly, to simplify the mechanism, the decomplexation of intermediates from the mixed acyl complex after the hydrolysis step was assumed to occur rapidly. Subsequently, the rate expressions for producing intermediates were simplified and the rate constants involving the model could be reduced. Similar rate expressions have been successfully applied in the hydrolysis reaction of triolein (Hermansyah et al., 2006) but not yet applied in the transesterification reaction. The conceptual scheme of the overall reaction mechanism is shown in Figure 3-12.

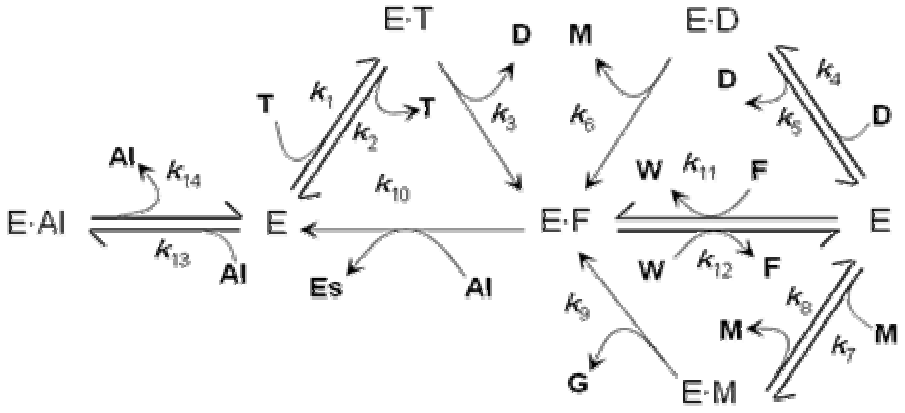


Figure 3-12 Conceptual scheme of overall reaction mechanism 2.

The development of the rate expressions for mechanism 2 follows the same general procedure as that for mechanism 1. The resultant rate expressions for mechanism 2 are:

$$\frac{d[T]}{dt} = -V_{mT}[T][E^*] \quad (24)$$

$$\frac{d[D]}{dt} = (V_{mT}[T] - V_{mD}[D])[E^*] \quad (25)$$

$$\frac{d[M]}{dt} = (V_{mD}[D] - V_{mM}[M])[E^*] \quad (26)$$

$$\frac{d[F]}{dt} = -\frac{d[W]}{dt} = (V_{mT}[T] + V_{mD}[D] + V_{mM}[M])\frac{V_{mF}[W]}{V_{mF}[W] + V_{eEs}[Al]}[E^*] + V_{rF}[F]\left(\frac{V_{mF}[W]}{V_{mF}[W] + V_{eEs}[Al]} - 1\right)[E^*] \quad (27)$$

$$\frac{d[Es]}{dt} = -\frac{d[Al]}{dt} = (V_{mT}[T] + V_{mD}[D] + V_{mM}[M] + V_{rF}[F])\frac{V_{eEs}[Al]}{V_{mF}[W] + V_{eEs}[Al]}[E^*] \quad (28)$$

The $[E^*]$ in Eqs. (24-28) is

$$[E^*] = [E_T]/\left(1 + [T]\left(K_{mT} + \frac{V_{mT}}{V_{mF}[W] + V_{eEs}[Al]}\right) + [D]\left(K_{mD} + \frac{V_{mD}}{V_{mF}[W] + V_{eEs}[Al]}\right) + [M]\left(K_{mM} + \frac{V_{mM}}{V_{mF}[W] + V_{eEs}[Al]}\right) + \frac{V_{rF}[F]}{V_{mF}[W] + V_{eEs}[Al]} + \frac{Al}{K_I}\right) \quad (29)$$

where V_{mT}, V_{mD}, V_{mM} and V_{eEs} are defined as:

$$V_{mT} = \frac{k_3 k_1}{k_2 + k_3},\ V_{mD} = \frac{k_6 k_4}{k_5 + k_6},\ V_{mM} = \frac{k_9 k_7}{k_8 + k_9} \text{ and } V_{eEs} = k_{10}.$$

V_{mF} and V_{rF} are rate constants of hydrolysis and re-esterification, respectively, for $E{\cdot}F$. They are defined as:

$$V_{mF} = k_{12} \text{ and } V_{rF} = k_{11}.$$

K_{mT}, K_{mD}, K_{mM} and K_I are

$$K_{mT} = \frac{k_1}{k_2 + k_3},\ K_{mD} = \frac{k_4}{k_5 + k_6},\ K_{mM} = \frac{k_7}{k_8 + k_9} \text{ and } K_I = \frac{k_{14}}{k_{13}}.$$

In this mechanism there are 10 unknown parameters so fewer than those in mechanism 1. These parameters were estimated by fitting the model equations with the experimental data obtained with various ethanol concentrations as parameters in mechanism 1.

Mechanism 3

Mechanism 3 differs from mechanisms 1 and 2 at the point in the kinetic reactions at which the molecule of ethanol engages. Since it was reported that it was more accurate to assume that transesterification takes place by direct alcoholysis of the triacylglycerols than two consecutive steps of hydrolysis and esterification (Al-Zuhair et al., 2007), in mechanism 3 the catalytic reaction was divided into two patterns of reaction; one represents the hydrolysis step to produce free fatty acid prior to the esterification step and another represents the ethanolysis reaction to directly produce the fatty acid ethyl ester. In practice, these two steps occur simultaneously.

Thus, the reaction network consists of a combination of a series of parallel reactions for each acylglycerol complex (*E·T*, *E·D* and *E·M*). The conceptual scheme of the overall reaction mechanism is shown in Figure 3-13.

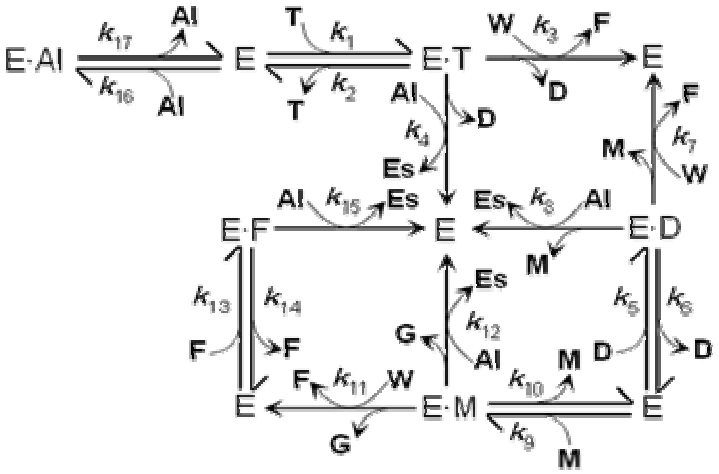


Figure 3-13 Conceptual scheme of overall reaction mechanism 3.

The development of the rate expressions for mechanism 3 also follows the same general procedure as that for mechanisms 1 and 2. The resultant rate expressions for mechanism 3 are:

$$\frac{d[T]}{dt} = -(V_{mT}[W] + V_{eT}[Al])[T][E^*] \tag{30}$$

$$\frac{d[D]}{dt} = ((V_{mT}[W] + V_{eT}[Al])[T] - (V_{mD}[W] + V_{eD}[Al])[D])[E^*] \tag{31}$$

$$\frac{d[M]}{dt} = ((V_{mD}[W] + V_{eD}[Al])[D] - (V_{mM}[W] + V_{eM}[Al])[M])[E^*] \tag{32}$$

$$\frac{d[G]}{dt} = (V_{mM}[W] + V_{eM}[Al])[M][E^*] \tag{33}$$

$$\frac{d[F]}{dt} = ((V_{mT}[T] + V_{mD}[D] + V_{mM}[M])[W] - V_{eEs}[F][Al])[E^*] \tag{34}$$

$$\frac{d[W]}{dt} = -(V_{mT}[T] + V_{mD}[D] + V_{mM}[M])[W][E^*] \tag{35}$$

$$\frac{d[Es]}{dt} = -\frac{d[Al]}{dt} = (V_{eT}[T] + V_{eD}[D] + V_{eM}[M] + V_{eEs}[F])[Al][E^*] \tag{36}$$

The $[E^*]$ in Eqs. (30-36) is

$$[E^*] = \frac{[E_T]}{1 + K_{mT}[T] + K_{mD}[D] + K_{mM}[M] + K_{mF}[F] + \dfrac{Al}{K_I}} \tag{37}$$

where V_{mT}, V_{mD}, V_{mM} and V_{eEs} are defined as:

$$V_{mT} = \frac{k_3 k_1}{k_2},\ V_{mD} = \frac{k_7 k_5}{k_6},\ V_{mM} = \frac{k_{11} k_9}{k_{10}} \text{ and } V_{eEs} = \frac{k_{15} k_{13}}{k_{14}} \quad ,$$

while V_{eT}, V_{eD} and V_{eM} are rate constants for ethanolysis of *T*, *D* and *M*, respectively. They are defined as:

$$V_{eT} = \frac{k_4 k_1}{k_2},\ V_{eD} = \frac{k_8 k_5}{k_6} \text{ and } V_{eM} = \frac{k_{12} k_9}{k_{10}}.$$

K_{mT}, K_{mD}, K_{mM}, K_{mF} and K_I are

$$K_{mT} = \frac{k_1}{k_2},\ K_{mD} = \frac{k_5}{k_6},\ K_{mM} = \frac{k_9}{k_{10}},\ K_{mF} = \frac{k_{13}}{k_{14}} \text{ and } K_I = \frac{k_{17}}{k_{16}}.$$

In this mechanism there are 12 unknown parameters. These parameters were estimated by fitting the model equations with the experimental data obtained under various ethanol concentrations just as was done for the parameters in mechanisms 1 and 2.

3.5.2 Determination of kinetic parameters

The concentrations of triacylglycerol, diacylglycerol, monoacylglycerol, free fatty acid and fatty acid ethyl ester at different times were obtained experimentally starting with 5 g of palm oil and various ethanol amounts of 0.29, 0.58 and 0.86 g to obtain molar ratios of ethanol to palm oil of 1, 2 and 3, respectively. The amounts of enzyme and water were 0.2 g and 10% based on the palm oil weight, respectively. The nonlinear curve fitting by Simplex's method (Nelder and Mead, 1964) was used for fitting the system of the differential equations for each mechanism into the experimental data. Simplex's method minimizes the sum of the squares of the difference between the experimental and calculated values. By fitting the above differential equations to the experimental data, the parameters involved in each model were estimated and are listed in Table 3-6.

Based upon these determined parameters and the kinetic scheme, the concentrations of each component in the different reactions could be calculated. Comparisons between the calculated and experimental data are presented in Figures 3-14, 3-15 and 3-16. To evaluate the suitability of the model, the sum of squares of the residuals in each model at the convergence step has been calculated as presented in Table 3-7. The consistently lowest values of the sum of squares of the residuals for each component in mechanism 3 reveal that the mechanism 3 is the most suitable one to be applied in simulation study than the other mechanisms.

Table 3-6 Parameters in the models

Parameters	Mechanism 1	Mechanism 2	Mechanism 3
Rate constants	($mmol^{-1}$ h^{-1})	(g^{-1} h^{-1})	($mmol^{-1}$ h^{-1})
V_{mT}	4.221	8.863	7.619×10^{-2}
V_{mD}	3.783	6.492	8.128×10^{-2}
V_{mM}	3.504	3.078	1.951×10^{-1}
V_{eEs}	22.731	5.594	1.383
V_{rT}	14.157	-	-
V_{rD}	11.313	-	-
V_{rM}	9.126	-	-
V_{mF}	-	5.172×10^{-2}	-
V_{rF}	-	2.281	-
V_{eT}	-	-	2.751
V_{eD}	-	-	1.176
V_{eM}	-	-	0.965
Equilibrium constants (g $mmol^{-1}$)			
K_{mT}	9.115×10^{-2}	9.120×10^{-2}	2.891×10^{-2}
K_{mD}	7.134×10^{-2}	8.532×10^{-2}	2.322×10^{-2}
K_{mM}	6.088×10^{-2}	7.425×10^{-2}	1.974×10^{-2}
K_{mF}	5.632×10^{-2}	-	1.121×10^{-2}
K_{mDF}	7.109×10^{-2}	-	-
K_{mMF}	5.989×10^{-2}	-	-
K_{mGF}	3.431×10^{-2}	-	-
Inhibition constant (mmol g^{-1})			
K_I	0.924	0.894	0.882

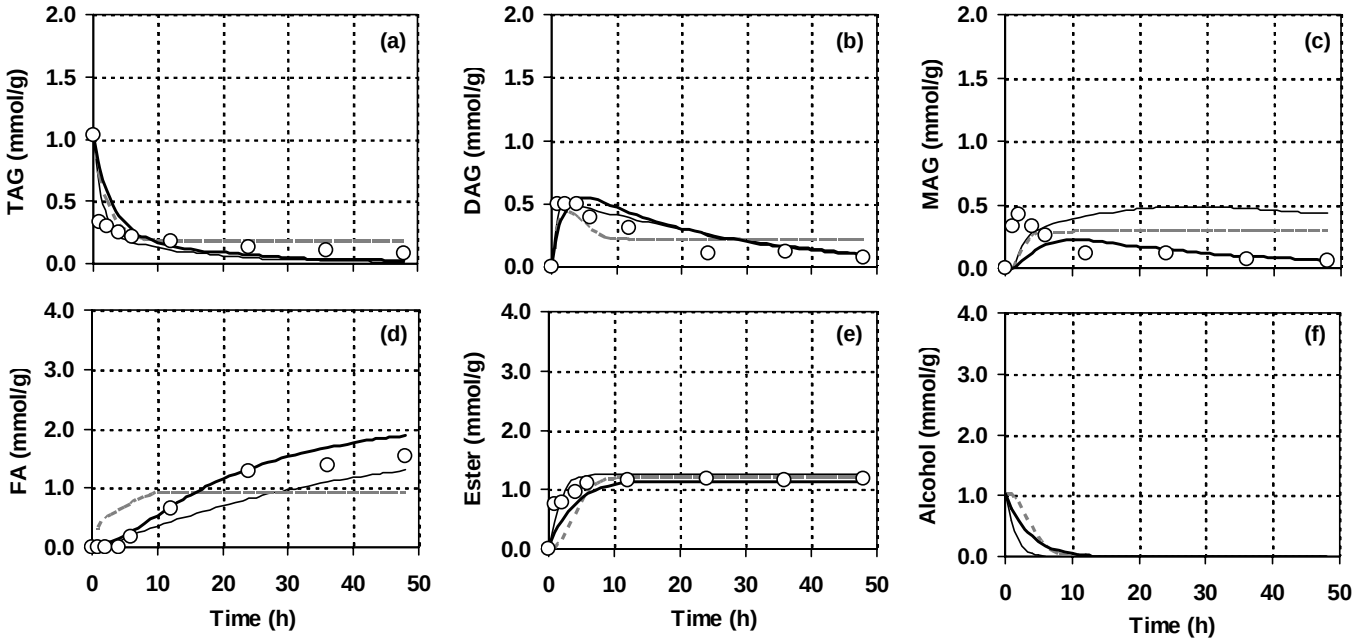


Figure 3-14 Comparison between calculated (lines) and experimental results (symbols) of intermediates changes including triacylglycerol (TAG), diacylglycerol (DAG), monoacylglycerol (MAG), free fatty acid (FA) and fatty acid ethyl ester (Ester) and calculated ethanol (Alcohol) concentrations in the transesterification reaction with ethanol to palm oil molar ratios of 1:1. Mechanism 1:——— ; Mechanism 2 - - - - ; Mechanism 3: ——— .

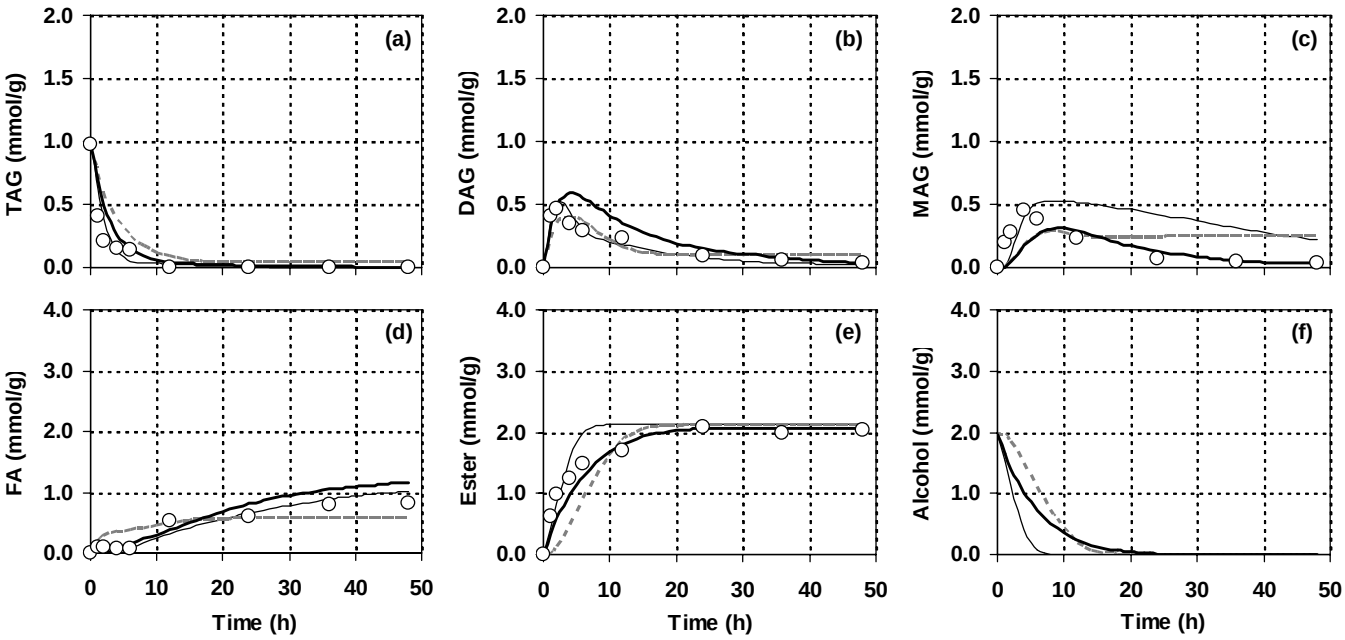


Figure 3-15 Comparison between calculated (lines) and experimental results (symbols) of intermediates changes including triacylglycerol (TAG), diacylglycerol (DAG), monoacylglycerol (MAG), free fatty acid (FA) and fatty acid ethyl ester (Ester) and calculated ethanol (Alcohol) concentrations in the transesterification reaction with ethanol to palm oil molar ratios of 2:1. Mechanism 1: ——; Mechanism 2: - - - - ; Mechanism 3: ——— .

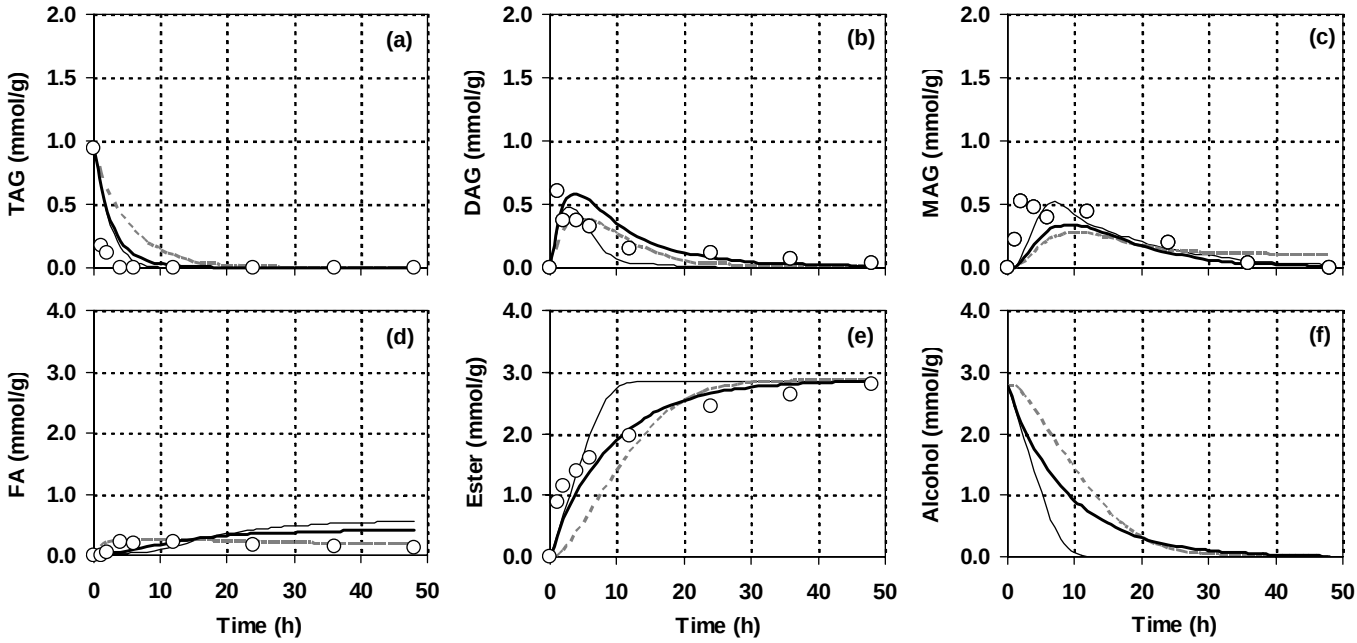


Figure 3-16 Comparison between calculated (lines) and experimental results (symbols) of intermediates changes including triacylglycerol (TAG), diacylglycerol (DAG), monoacylglycerol (MAG), free fatty acid (FA) and fatty acid ethyl ester (Ester) and calculated ethanol (Alcohol) concentrations in the transesterification reaction with ethanol to palm oil molar ratios of 3:1. Mechanism 1: —— Mechanism 2: - - - - ; Mechanism 3: ——— .

Table 3-7 Comparison of the sum of squares of the residuals in each model

Component	Sum of squares of the residuals		
	Mechanism 1	Mechanism 2	Mechanism 3
TAG	7.45	9.80	6.37
DAG	7.54	8.35	5.73
MAG	19.12	17.31	11.55
FA	13.09	9.03	8.14
Ester	3.84	10.20	2.59
Total sum of squares	51.03	54.69	34.39

TAG: triacylglycerol, DAG: diacylglycerol, MAG: monoacylglycerol, FA: free fatty acid, Ester: fatty acid ethyl ester.

A visual inspection of Figures 3-14, 3-15 and 3-16 also indicates that the mechanism 3 model provides a good approximation to most of the data sets. At a low ethanol concentration the simulated results of mechanisms 1 and 2 did not fit well to the experimental data especially for the monoacylglycerol concentration while at high ethanol concentrations both these mechanisms did not explain well the determined concentrations of the fatty acid ethyl ester. Thus, the concept that the two reactions of hydrolysis and transesterification occur simultaneously is more acceptable than the concept that acylglycerols are hydrolyzed and fatty acids become free prior to ethyl ester synthesis. This was confirmed by the experimental results that showed that the concentration of the measurable free fatty acid was always low during the time course of the reaction at a high initial ethanol concentration (Figure 3-16) indicating that in the presence of ethanol, the fatty acid in acylglycerol was directly used in ethanolysis reaction, rather than being released as a free fatty acid by the hydrolysis reaction.

In terms of the reaction rate constants of mechanism 3 in Table 3-6, the rate constants of triacylglycerol, diacylglycerol and monoacylglycerol in the hydrolysis reaction (V_{mT} = 0.07619 $mmol^{-1}h^{-1}$, V_{mD} = 0.08128 $mmol^{-1}h^{-1}$, V_{mM} = 0.1951 $mmol^{-1}h^{-1}$) were much lower than those in the ethanolysis reaction (V_{eT} = 2.751 $mmol^{-1}h^{-1}$, V_{eD} = 1.176 $mmol^{-1}h^{-1}$ and V_{eM} = 0.965 $mmol^{-1}h^{-1}$). This indicated that in the presence of ethanol the acylglycerols were more easily converted to fatty acid ethyl ester through ethanolysis reaction rather than to free fatty acid through hydrolysis reaction. This result also supports the assumption that transesterification of triacylglycerol occurs directly by the alcoholysis reaction as has been reported in the literature (Al-Zuhair et

al., 2007). However, in those studies, the behavior of the substrates and intermediates, namely acylglycerols and free fatty acids were not considered. The results presented in this paper also showed that the concentration of measurable intermediate free fatty acid was low during the time course of the reaction at high ethanol concentrations (Figure 3-16). This result implied that the ethanolysis reaction is dominant over the hydrolysis reaction at a high initial ethanol concentration. On the other hand, the hydrolysis reaction might require higher concentrations of water to increase the hydrolysis reaction rate. For a further study, the estimated parameters of mechanism 3 in Table 3-6 were used in computer simulations to investigate the sensitivity of the model and the impact of the ethanol concentration on the transesterification reaction of palm oil by the immobilized lipase.

3.5.3 Sensitivity of the model

A sensitivity analysis of the parameters in the model was carried out by calculating the initial production rates of fatty acid ethyl ester and free fatty acid under condition where the value of one parameter was changed without changing any other parameter. Table 3-8 shows the effects of parameters on the predicted initial production rates of fatty acid ethyl ester and free fatty acid by increasing or decreasing the parameter values by 50%. The results show that the changes of V_{eT}, V_{eD} and V_{eM} produced larger differences (2-31%) on the production rates of fatty acid ethyl ester than did V_{mT}, V_{mD}, and V_{mM} (0.01-0.35%). As the values of V_{eT}, V_{eD}, V_{eM}, and V_{eEs} increased by 50% the production rates of fatty acid ethyl ester also increased 0.88-19.4%, however, the concentration of fatty acid ethyl ester at equilibrium was not affected (data not shown). On the other hand, as the values of V_{mT}, V_{mD}, and V_{mM} increased by 50%, the production rates of free fatty acid increased 12-15%. This result confirmed that the parameters involved in the ethanolysis reaction (V_{eT}, V_{eD} and V_{eM}) had the most important role on the prediction of fatty acid ethyl ester production whereas the parameters involved in the hydrolysis reaction (V_{mT}, V_{mD} and V_{mM}) had the biggest effect on the prediction of free fatty acid production. In addition, the reaction rates increased when K_{mT}, K_{mD}, K_{mM} and K_{mF} were decreased. However, among the equilibrium constants, K_I produced the largest difference (20-46%) to the reaction rates. This confirmed that the model could be applied to predict the impact of the ethanol concentration on biodiesel production by the immobilized lipase.

Table 3-8 Sensitivity of kinetic parameters on initial production rates of fatty acid ethyl ester (r_{Es}) and free fatty acid (r_F)

Initial rate	Parameter change	Deviation of initial rate with parameter change (%)											
		V_{mT}	V_{mD}	V_{mM}	V_{eEs}	V_{eT}	V_{eD}	V_{eM}	K_{mT}	K_{mD}	K_{mM}	K_{mF}	K_I
r_{Es}	+50%	+0.35	+0.24	+0.01	+0.88	+19.4	+8.95	+1.81	-0.17	-0.13	-0.05	-0.01	+20.8
	-50%	-0.35	-0.25	-0.01	-1.14	-31.4	-12.1	-2.11	+0.17	+0.13	+0.03	+0.01	-34.2
r_F	+50%	+14.3	+21.2	+12.7	-14.4	+25.1	+6.22	-1.68	-0.28	-0.23	-0.05	-0.01	+38.6
	-50%	-14.9	-22.3	-13.8	+18.8	-23.3	-7.09	+1.87	+0.28	+0.22	+0.04	+0.01	-46.7

+, - : The values increase or decrease from the values without parameter change.

3.5.4 Simulations

Because no solvent was used in this system, it was physically impossible to change the concentration of one reactant without changing that of the others. However, computer simulations allow one to independently assess the effects of hypothetical changes in the concentrations of each substrate and intermediate. Furthermore, the computer simulations also permit one to examine the dynamics and equilibria of the transesterification reaction that cannot be investigated experimentally. Beyond these limitations, the model based on mechanism 3 provides a useful description of the transesterification of palm oil with ethanol by immobilized lipase. The estimated parameters were considered to be the most representative of the palm oil data. The effects of changes in the value of the initial concentrations of alcohol were investigated in a series of simulations. The calculated results using the model of mechanism 3 are shown in Figure 3-17. The initial ethanol concentration was varied to obtain molar ratios of ethanol to palm oil in the range of 0.5 to 6 while all other initial conditions (water 10% based on oil weight and enzyme amount 0.2 g) were held constant.

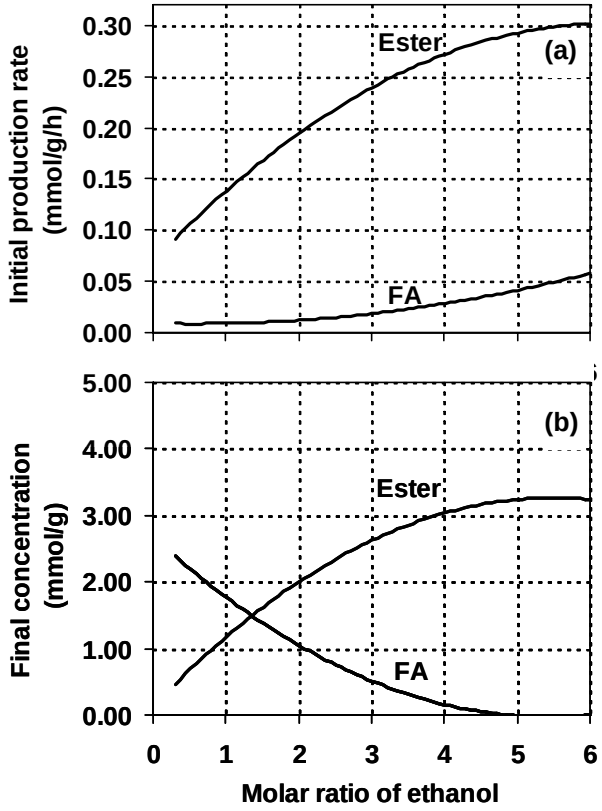


Figure 3-17 Simulation results of the effects of molar ratio of ethanol on the initial production rates (a) and final concentrations (b) of fatty acid ethyl ester (Ester) and free fatty acid (FA).

Figure 3-17a indicates that higher initial concentration of ethanol lead to a faster initial rate of fatty acid ethyl ester production. The result is expected from a kinetic point of view, because a higher concentration of ethanol could enhance the production rate of the fatty acid ethyl ester. Hence, much higher ethanol concentrations would be required for inhibition to take place in a solvent free system. By comparing the initial rates of fatty acid ethyl ester and free fatty acid production as shown in Figure 3-17a, the initial rates of fatty acid ethyl ester production were found to be much higher than those of free fatty acid production. This indicated that ethanol is incorporated faster than is water as was also indicated by the higher values of the rate constants involving the ethanolysis reaction than those in the hydrolysis reaction (Table 3-6). It was also found that the production rates of free fatty acid also increased with increasing ethanol concentrations. This might be due to the thermodynamic shift

from monoacylglycerol to free fatty acid when a high concentration of monoacylglycerol was obtained from the ethanolysis reaction.

In addition, the higher initial concentrations of ethanol resulted in greater amounts of ethanol being incorporated into the fatty acid of palm oil (at equilibrium) as shown in Figure 3-17b. From a thermodynamic standpoint, a greater incorporation of ethanol is expected because a higher concentration of this ethanol should shift the equilibrium toward greater transesterification. However, a molar ratio of ethanol to palm oil higher than 4, resulted only a slight increase in the final concentration of fatty acid ethyl ester. Theoretically transesterification of one mole of triacylglycerol needs 3 moles of ethanol, however the yields of fatty acid ethyl ester depend on the preferred equilibrium in various conditions. The simulation results also show that increasing the initial ethanol concentration produces a lowering of the final concentration of fatty acid due to the thermodynamic equilibrium shift to transesterification. On the other hand, higher yield of free fatty acid was produced at a lower ethanol concentration.

3.6 Continuous transesterification of oil to ethanol

Continuous transesterification of palm oil on a packed bed reactor was also investigated. The optimal flow rate was determined using the refined palm oil and the continuous transesterification of refined, crude and used palm oil were compared.

3.6.1 Effect of flow rate

The flow rate of oil is an important parameter in continuous reaction in the packed bed reactor. If the flow rate is too high, the contact time of oil on immobilized lipase will be too short and the reaction will be incomplete. On the other hand, if the flow rate is too low, the productivity will be too low. The effect of flow rate on conversion of FAEE from refined palm oil was summarized in Table 3-9. The mixture of oil and ethanol were contained in a screw-top bottle and stirred magnetically at 45˚C in a water bath and circulated using a peristaltic pump through a column (inside diameter 1 cm, height 10 cm) containing immobilized lipase on Accurel EP-100. When only the immobilized lipase on small particle Accurel EP-100 (<400 μm) was packed into the column, the column clogging was observed in 8 h of running time. In order to solve this problem, the immobilized lipase was mixed with the larger size of Accurel EP-100 (1000-1500 μm) in a 2:1 ratio before filling a column so as to increase the system porosity (H-Kittikun et al., 2000). From Table 3-9, it was found that when the flow rate of substrate increased the conversion of FAEE decreased. The optimal flow rate which gave the highest productivity of 0.111 g/min and comparable high conversion of FAEE 38.25 % was at 0.29 g/min.

Table 3-9 Effect of flow rate on conversion of FAEE and productivity

Flow rate (g/min)	Conversion of FAEE (%)	Productivity of FAEE[a] (g/min)
0.19	42.47	0.081
0.24	40.77	0.098
0.29	38.25	0.111
0.33	33.49	0.111

[a] – Productivity of FAEE = conversion of FAEE × flow rate.

3.6.2 Long-term operation

The optimal substrate flow rate for FAEE production was 0.29 g/min. This flow rate was used for long-term continuous FAEE production in the packed-bed reactor. The results are shown in Figure 3-18. It was found that the reactor could be successfully operated and maintained at average 34% yield of FAEE for 90 h without pressure drops problem. The highest FAEE yield of 37% was obtained at 18 h and nearly stable for 32 h. Subsequently, the FAEE yield was slowly decreased with increasing the operation time. On the other hand, FFA yield was nearly 0% through out the operation. A productivity of 142 g FAEE/day was obtained for continuous operation.

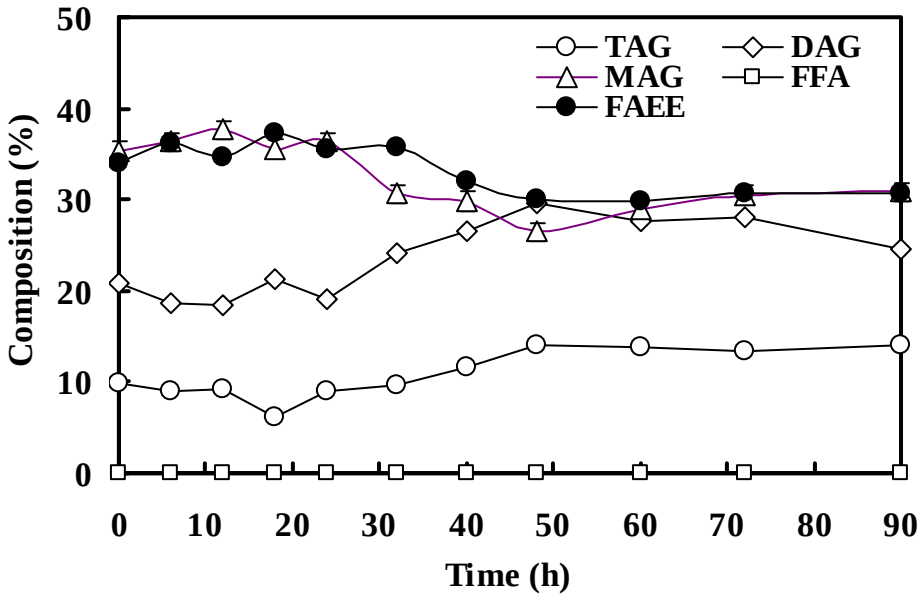


Figure 3-18 Long-term operation of continuous FAEE production in a packed-bed reactor.

CHAPTER 4 Conclusions and Suggestions

The findings of this study have shown that lipases from *Pseudomonas* sp. (lipase PS) immobilized on Accurel are able to catalyze transesterification of palm oil with appropriated ethanol and methanol for FAEE and FAME production, respectively. The kinetics of a lipase-catalyzed transesterification of triacylglycerol with ethanol for biodiesel production were successfully modeled using rate expressions requiring adjustable parameters. The mechanism for transesterification by the immobilized lipase was determined by the construction of various mechanisms in the models and then fitted to the experimental data. The simulation results indicated that the two reactions of hydrolysis and ethanolysis occurred simultaneously rather than by the stepwise hydrolysis followed by esterification. Furthermore, unlike previous reports in the literature, the present model was able to account for the effects of concentrations of all chemical species participating in the transesterification reaction throughout the entire reaction especially the impact of the ethanol concentration. From a thermodynamic standpoint, a greater incorporation of ethanol is expected because a higher concentration of this acyl acceptor should shift the equilibrium towards a faster transesterification reaction. However, higher ethanol concentrations could inhibit the reaction. Hence, the model that included the inhibition by ethanol could predict the appropriate conditions for the efficient production of the fatty acid ethyl ester. In the continuous production of FAEE in a packed-bed reactor, the yield of FAEE increased with decreasing in the flow rate of substrate. At the substrate flow rate of 0.29 g/min gave FAEE yield of 38.25 % and the highest productivity of FAEE of 0.111 g/min. Furthermore, the long term operation of the continuous process gave the average yield of FAEE at 34% for 90 h with the productivity of 142g FAEE/day. However, further study on the improvement of FAEE yield in the continuous process and the stability of immobilized lipase and scale-up of continuous FAEE production is necessary.

References

Abigor, R.D., Uadia, P.O., Foglia, T.A., Haas, M.J., Jones, K.C., Okpefa, E., Obibuzor, J.U. and Bafor, M.E. (2000) Lipase-catalysed production of biodiesel fuel from some Nigerian lauric oils. Biochem. Soc. Transac. 28(6), 979–981.

Al-Zuhair, S. (2005) Production of biodiesel by lipase-catalyzed transesterification of vegetable oils: a kinetic study. Biotechnol. Prog. 21(5), 1442-1448.

Al-Zuhair, S., Ling, F.W. and Jun, L.S. (2007) Proposed kinetic mechanism of the production of biodiesel from palm oil using lipase. Process Biochem. 42, 951-960.

Brady, C., Metcalfe, L., Slaboszewski, D. and Frank, D. (1988) Lipase immobilized on a hydrophobic microporous supports for the hydrolysis of fats. J. Am. Oil Chem. Soc. 65, 917-921.

Brzozowski, A.M., Derewenda, U., Derewenda, Z.S., Dodson, G.G., Lawson, D.M., Turkenburg, J.P., Björkling, F., Huge-Jensen, B., Patkar, S.A. and Thim, L. (1991) A model for interfacial activation in lipases from the structure of a fungal lipase-inhibitor complex. Nature. 351, 491-494.

Cheirsilp, B., Kaewthong, W. and H-Kittikun, A. (2007) Kinetic study of glycerolysis of palm olein for monoacylglycerol production by immobilized lipase. Biochem. Eng. J. 35(1), 71-80.

Chen, J.P. and Wang, H.Y. (1998) Improved properties of bilirubin oxidase by entrapment in alginate-silicate sol-gel matrix. Biotech. Technol. 12(11), 851-853.

Chen, J.W and Wu, W.T. (2003) Regeneration of immobilized *Candida antarctica* lipase for transesterification. J. Biosci. Bioeng. 95(5), 466-469.

Crabbe, E., Nolasco-Hipolito, C., Kobayashi, G., Sonomoto, K., and Ishizaki, A. (2001) Biodiesel production from crude palm oil and evaluation of butanol extraction and fuel properties. Process Biochem. 37, 65–71.

Danby, J.M.A. Computer modeling, Willmann-Bell Inc Richmond Va 1997.

Dossat, V., Combes, D. and Marty, A. (2002) Lipase-catalysed transesterification of high oleic sunflower oil. Enzyme Microb. Technol. 30, 90-94.

Du, W., Xu, Y., Liu, D. and Zeng, J. (2004) Comparative study on lipase-catalyzed transformation of soybean oil for biodiesel production with different acyl acceptors. J. Mol. Catal. B: Enzym. 30, 125-129.

Gandhi, N.N. (1997) Application of lipase. J. Am. Oil Chem. Soc. 74(6), 621-634.

Ghosh, S. and Bhattacharyya, D.K. (1995) Utilization of acid oils in making valuable fatty products by microbial lipase technology. J. Am. Oil Chem. Soc. 72, 1541-1544.

Godtfredsen, S.E. (1993) Lipase. *In* Enzymes in Food Processing. 3rd ed. (Nagodawithana, T. and Reed, G., eds.). p. 205-219. Academic Press. California.

Gunstone, F.D. (1997) Major Sources of Lipids. *In* Lipid Technologies and Applications. (Gunstone, F.D. and Padley, F.B., eds.). p. 834. Marcel Dekker. New York.

Hermansyah, H., Kubo, M., Shibasaki-Kitakawa, N. and Yonemoto, T. (2006) Mathematical model for stepwise hydrolysis of triolein using *Candida rugosa* lipase in biphasic oil-water system. Biochem. Eng. J. 31, 125-132.

H-Kittikun, A., Prasertsan, P. and Sungpud, C. (2000) Continuous production of fatty acids from palm olein by immobilized lipase in a two-phase system. J. Am. Oil Chem. Soc. 77(6), 599-603.

Kaieda, M., Samukawa, T., Kondo, A. and Fukuda, H. (2001) Effect of methanol and water contents on production of biodiesel fuel from plant oil catalyzed by various lipases in a solvent-free system. J. Biosci. Bioeng. 91(1), 12-15.

Kaieda, M., Samukawa, T., Matsumoto, T., Ban, K., Kondo, A., Shimada, Y., Noda, H., Nomoto, F., Ohtsuka, K., Izumoto, E. and Fukuda, H. (1999) Biodiesel fuel production from plant oil catalyzed by *Rhizopus oryzae* lipase in a water-containing system without an organic solvent. J. Biosci. Bioeng. 88(6), 627-631.

Kalam, M.A. and Masjuki, H.H. (2002) Biodiesel from palm oil—an analysis of its properties and potential. Biomass Bioenergy. 23, 471 – 479.

Krisnangkura, K. and Simamaharnnop, R. (1992) Continuous transmethylation of palm oil in an organic solvent. J. Am. Oil Chem. Soc. 69, 166-169.

Lee, S.Y. and Rhee, J.S. (1993) Production and partial purification of a lipase from *Pseudomonas putida* 3SK. Enzyme Microb. Technol. 15, 617–623.

Li, L., Du, W., Liu, D., Wang, L. and Li, Z. (2006) Lipase-catalyzed transesterification of rapeseed oils for biodiesel production with a novel organic solvent as the reaction medium. J. Mol. Catal. B: Enzym. 43, 58-62.

Lortie, R., Trani, M. and Ergan, F. (1993) Kinetic study of the lipase-catalyzed synthesis of triolein. Biotechnol. Bioeng. 41, 1021-1026.

Malcata, F.X., Reyes, H.R., Garcia, H.S., Jr. Hill, C.G.. and Amundson, C.H. (1992) Kinetics and mechanisms of reactions catalyzed by immobilized lipases. Enzyme Microb. Technol. 14, 426-446.

Meher, L.C., Vidya Sagar, D. and Naik, S.N. (2006) Technical aspects of biodiesel production by transesterification-a review. Renewable and Sustainable Energy Reviews. 10, 248–268.

Montero, S., Blanco, A., Virto, M.D., Landeta, L.C., Agud, I. and Solozabal, R. (1993) Immobilization of *Candida rugosa* lipase and some properties of the immobilized enzyme. Enzyme Microb. Technol. 15(3), 239–47.

Murray, M., Rooney, D., Van Neikerk, M., Montenegro, A. and Weatherley, L. R. (1997) Immobilization of lipase onto lipophilic polymer particles and application to oil hydrolysis. Process Biochem. 32(6), 479-486.

Nelder, J.A. and Mead, R. (1964) A simplex method for function minimization, Comput. J. 7, 308-313.

Nelson, L.A., Foglia, T.A. and Marmer, W.N. (1996) Lipase-catalyzed production of biodiesel. J. Am. Oil Chem. Soc. 73(8), 1191-1195.

Noureddini, H., Gao, X. and Philkana, R.S. (2005) Immobilized *Pseudomonas cepacia* lipase for biodiesel fuel production from soybean oil. Bioresour. Technol. 96, 769–777.

Padmini, P., Rakshit, S.K. and Baradarajan, A. (1994) Kinetics of enzymatic hydrolysis of rice bran oil in organic system. Enzyme Microb. Technol. 16, 432-435.

Piyatheerawong, W., Iwasaki, Y., Xu, X. and Yamane, T. (2004) Dependency of water concentration on ethanolysis of trioleoyglycerol by lipases. J. Mol. Catal. B: Enzym. 28, 19-24.

Reyes, H.R. and Hill, C.G. (1994) Kinetic modeling of interesterification reactions catalyzed by immobilized lipase. Biotechnol. Bioeng. 43, 171-182.

Romero, M.D., Calvo, L., Alba, C. and Daneshfar, A. (2007) A kinetic study of isoamyl acetate synthesis by immobilized lipase-catalyzed acetylation in *n*-hexane. J. Biotech. 127, 269–277.

Rosu, R., Vozaki, Y., Iwasaki, Y. and Yamane, T. (1997) Repeated use of immobilized lipase for monoacylglycerol production by solid-phase glycerolysis of olive oil. J. Am. Oil Chem. Soc. 74(4), 445–450.

Salis, A., Pinna, M., Monduzzi, M. and Solinas, V. (2005) Biodiesel production from triolein and short chain alcohols through biocatalysis. J. Biotech. 119, 291–299.

Salis, A., Sanjust, E., Solinas, V. and Monduzzi, M. (2003) Characterisation of Accurel MP1004 polypropylene powder and its use as a support for lipase immobilization. J. Mol. Catal. B: Enzym. 24–25, 75–82.

Shah, S. and Gupta, M.N. (2007) Lipase catalyzed preparation of biodiesel from *Jatropha* oil in a solvent free system. Process Biochem. 42, 409–414.

Shimada, Y., Watanabe, Y., Samukawa, T., Sugihara, A., Noda, H., Fukuda, H. and Tominaga, Y. (1999) Conversion of vegetable oil to biodiesel using immobilized *Candida antarctica* lipase. J. Am. Oil Chem. Soc. 76 (7), 789–793.

Shimada, Y.,Watanabe, Y., Sugihara, A. and Tominaga, Y. (2002) Enzymatic alcoholysis for biodiesel fuel production and application of the reaction to oil processing. J. Mol. Catal. B: Enzym. 17, 133–142.

Soumanou, M.M. and Bornscheuer, U.T. (2003) Improvement in lipase-catalyzed synthesis of fatty acid methyl esters from sunflower oil. Enzyme Microb. Technol. 33, 97-103.

Talukder, R., Mahabubur, Md., Sze, M.P., Jin, C.W., Choi, J.W. and Yvonne, C. (2006) Lipase-catalyzed methanolysis of palm oil in presence and absence of organic solvent for production of biodiesel. Biocatalysis and Biotransformation. 24(4), 257-262.

Taylor, F., Kurantz, M.J. and Craig, J.C. (1992) Kinetics of continuous hydrolysis of tallow in a multi-layered flat-plate immobilized-lipase reactor. J. Am. Oil Chem. Soc. 69 (6), 591-594.

Tueter, M., Aksoy, H.A., Gilbaz, E.E. and Kursun, E. (2004) Synthesis of fatty acid esters from acid oils using lipase B from *Candida antarctica*. Eur. J. Lipid Sci. Technol. 106, 513-517.

Watanabe, Y., Pinsirodom, P., Nagao, T., Yamaguchi, A., Kobayashi, T. Nishida, Y., Takagi, Y. and Shimada, Y. (2007) Conversion of acid oil by-produced in vegetable oil refining to biodiesel fuel by immobilized *Candida antarctica* lipase. J. Mol. Catal. B: Enzym. 44, 99-105.

Watanabe, Y., Shimada, Y., Sugihara, A., Noda, H., Fukuda, H. and Tominaga, Y. (2000) Continuous production of biodiesel fuel from vegetable oil using immobilized *Candida antarctica* lipase. J. Am. Oil Chem. Soc.77 (4), 355–360.

Xu, Y., Du, X. and Liu, D. (2005) Study on the kinetics of enzymatic interesterification of triglycerides for biodiesel production with methyl acetate as the acyl acceptor. J. Mol. Catalysis B: Enzymatic 32, 241-245.

Yamada, H., Sorimachi, Y. and Tagawa, T. (2007) Operation optimization of lipase-catalyzed biodiesel production. J. Chem. Eng. Japan. 40(7), 571-574.

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Appendix

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Impact of transesterification mechanisms on the kinetic modeling of biodiesel production by immobilized lipase

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ABSTRACT

Three kinetic models of the transesterification of palm oil fatty acids to ethanol using an immobilized lipase were developed. The models differ from one another with respect to the rate-limiting step and the point at which the ethanol molecule becomes involved in the reaction. The kinetic parameters were estimated by fitting experimental data of the transesterification of palm oil with various ethanol concentrations. The models are able to account for the effects of substrates and products involved in the transesterification throughout the entire reaction. There was a good agreement between experimental results and those predicted by the proposed model equations in which ethanol was assumed to be involved directly in an alcoholysis reaction with palm oil. Furthermore, the calculated results show that the rate constants for alcoholysis of palm oil with ethanol are much higher than those for the hydrolysis reaction. From the proposed model equations, the effects of ethanol concentration on the initial production rates and yields of fatty acid ethyl ester and free fatty acids were simulated. The simulation results show that increasing the initial ethanol concentration produces an increase in the initial production rate and yield of fatty acid ethyl ester and lowers the final concentration of free fatty acid whereas lower ethanol concentration led to a higher final concentration of free fatty acid.

1. Introduction

Fatty acid alkyl esters derived from natural fats and oils have recently attracted much research interest because of their value as a source of biodiesel. They are a renewable source while the availability of petroleum chemicals is finite. Fatty acid alkyl esters are produced by the transesterification of triglycerides to alcohols preferentially methanol and ethanol. Biodiesel from palm oil can be domestically produced in Thailand, offering the possibility of reducing petroleum imports. The preferred industrial process for producing biodiesel from palm oil typically uses an alkaline or acid catalyzed transesterification reaction [1–3]. However, the undesired side reaction, saponification, occurs and an extra separation step is required to remove the homogeneous catalysts, thus inflating the cost of production.

Recently, utilization of a lipase to produce these fatty acid alkyl esters has been shown to be effective [4–9]. Several researchers have investigated biodiesel production from palm oil using immobilized lipases [10,11]. However, these studies focused on a series of experiments to optimize production while the dynamics and equilibrium of the enzymatic reaction could not be investigated experimentally. In order to identify the optimal conditions for the lipase catalyzed transesterification reaction, it is essential to understand the kinetics of this reaction. Until now all kinetic mechanisms on lipase catalytic reactions have been mostly based on the hydrolysis of triacylglycerol or the esterification of fatty acid [12–17]. Only a small number of kinetic studies on the transesterification of oils by immobilized lipases have been found in the literature [18–20]. In addition, the transesterification by immobilized lipase was assumed to take place in two consecutive hydrolysis and esterification steps [18–19]. In this two-step process, triacylglycerols were first hydrolyzed to produce free fatty acids and then the free fatty acids were esterified to produce new esters. Some authors however have assumed that the transesterification takes place by direct alcoholysis of the triglycerides [20]. Unfortunately, clarification of the mechanisms and the corresponding rate expressions of the transesterification reactions have not been conducted.

To elucidate the mechanism of transesterification of two substrates, namely triacylglycerol and alcohol, computer simulation could be a useful tool to independently assess the effects of hypothetical changes in the concentrations of each substrate. Furthermore, it is important to emphasize the effect of alcohol concentration on the mechanism of transesterification since it was

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reported that alcohol had an inhibitory effect on fatty acid alkyl ester production [4,21]. Although methanol is easily available as an absolute alcohol, it is also involved in enzyme inactivation processes [22]. Ethanol is environmentally attractive because unlike methanol, ethanol could be produced from renewable resources and used in biodiesel production [23]. Also, ethanol is a better solvent than methanol for oil [5]. In this study, palm oil and ethanol were used as reactants. The kinetics of transesterification of palm oil and ethanol for fatty acid ethyl ester production was investigated. First, a simple model based on Ping-Pong Bi Bi was proposed to describe the kinetics of the transesterification and hydrolysis reactions. Then, the mathematical models for the reactions, taking into account the effect of ethanol concentration, were considered. Unlike previous reports in the literature, the present models are able to account for the effects of the concentrations of all chemical species participating in the transesterification reaction. In addition, the effects of ethanol concentrations on the production of fatty acid ethyl ester were incorporated into the model.

2. Materials and methods

2.1. Materials

Lipase PS (*Pseudomonas* sp.) was a gift from Amano Pharmaceutical Co. Ltd., Japan. Microporous polypropylene powder; Accurel EP-100 (particle size 200–400 μm) was a gift from Akzo Nobel (Obermburg, Germany). Palm oil was purchased from Morakot Industry Co. Ltd., Thailand and it contained 96.07% of triacylglycerol and 3.93% of diacylglycerol. The composition of fatty acids in palm oil was 43.70% of palmitic acid, 45.76% of oleic acid, 8.35% of linoleic acid and 2.19% of palmitoleic acid. All other chemicals were analytical grade reagents obtained from commercial sources.

2.2. Immobilization

The procedure for immobilizing the enzyme was as described in the previous report [24]. Accurel EP-100 (0.2 g) was added to 20 ml of 0.1 M phosphate buffer (pH 7) containing approximately 50 U/ml lipase PS and the reaction mixture was stirred with a magnetic bar at 100 rpm for 30 min. Then, the suspension was filtered through a filter paper by vacuum. The immobilized enzyme was washed on the filter paper with 5 ml of 0.1 M phosphate buffer (pH 7) to remove soluble enzyme and dried in a vacuum desiccator. The immobilized lipase PS on Accurel EP-100 (250 U/g) was stored at 4 °C for further studies. The stability of the immobilized lipase PS has been studied in a previous report [24]. It was found that more than 80% of the hydrolytic activity remained after being incubated at 45 °C for 24 h.

2.3. Transesterification of palm oil for fatty acid ethyl ester production

The transesterification reactions were carried out in a batch system. The reaction mixture consisted of palm oil, enzyme, water and various ethanol concentrations. The ethanol was first mixed with palm oil to avoid the denaturation of the immobilized lipase by ethanol. The temperature was controlled at 45 °C. The reaction mixture was stirred at 500 rpm. Samples of the reaction mixture were centrifuged to remove immobilized lipase before analysis.

2.4. Analytical method

The components of the oil phase were analyzed for fatty acid ethyl ester, triacylglycerol, diacylglycerol, monoacylglycerol and free fatty acid using thin-layer chromatography with a flame ionization detection system (TLC/FID) (IATROSCAN MK5, Iatron Laboratories Inc. Tokyo, Japan) [25]. In this experiment, the percentage of the peak area was assumed to be the percent content of the corresponding compound [26]. The fatty acid compositions of triacylglycerols were determined by converting all fatty acids of triacylglycerols into the corresponding fatty acid methyl esters followed by gas chromatography (GC) analysis [27]. The lipase activity was determined by the modified cupric acetate method [28]. One unit of hydrolytic activity was defined as the amount of the enzyme, that liberated 1 μmol equivalent of palmitic acid from palm oil in 1 min at 45 °C.

All the data presented in this paper for reactant and product concentrations under various conditions were the means of two experiments with <10% deviation.

Ordinary differential equations were solved by the Runge-Kutta single-step fourth-order method [29]. The programs were coded in the Visual Basic program ver. 6.0 (Microsoft Inc., USA).

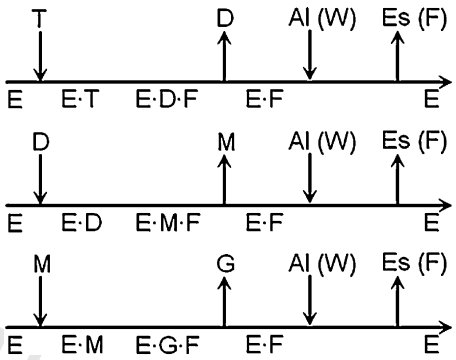

Fig. 1. Schematic diagram of Ping Pong Bi Bi mechanisms for stepwise transesterification of palm oil.

3. Results and discussion

3.1. Modeling of the lipase catalyzed transesterification reaction

Mechanisms of lipase catalyzed transesterification reaction based on hydrolysis and esterification of triacylglycerol, diacylglycerol, monoacylglycerol, free fatty acid and ethanol were considered. The starting point for the development of an appropriate generic mechanism describing the transesterification reactions is the mechanism of the ester bond hydrolysis reaction to produce free fatty acid, which constitutes the first step. This ester hydrolysis step is followed by the esterification step that produces a new ester bond by reacting the newly created free fatty acid with the incoming alcohol group. Fig. 1 shows the hydrolysis and esterification steps with the free enzyme (*E*) reacting with triacylglycerol (*T*) to form the first complex (*E·T*) and then *T* is hydrolyzed to diacylglycerol (*D*) and fatty acid (*F*). Subsequently, *D* is released from the second complex (*E·D·F*) to form the third complex (*E·F*). This complex might react with alcohol (Al) through an alcoholysis reaction to form an alkyl ester (Es) or with water (*W*) through a hydrolysis reaction to form free *F*. The mechanisms for the hydrolysis of *D* and monoacylglycerol (*M*) are also similar to that described above. However, the precise point in the kinetic cycle at which the ethanol molecule enters the active site is unknown, therefore three mechanisms were proposed. The models differ from one another with respect to the rate-limiting step and the point at which the ethanol molecule enters the catalytic cycle. Rate expressions corresponding to these mechanisms are developed and tested for consistency with the experimental results with various ethanol concentrations. Three mechanisms

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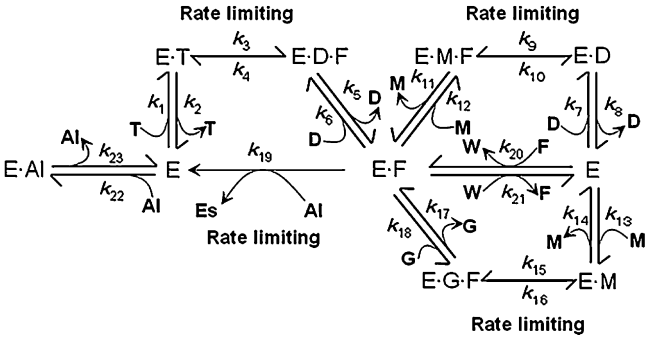

Fig. 2. Conceptual scheme of overall reaction mechanism 1.

share three basic assumptions: (1) since preliminary experimental data indicated that the reaction rate is sufficiently slow so that the mass transfer limitations imposed by the porous support are negligible. This has been also reported in the literature [16,30,31], the possible mass transfer limitations in this reaction system were ignored; (2) all the fatty acids released from the triacylglycerol substrate may be lumped together and treated as a single constituent (*F*); (3) the inhibition of enzyme activity by alcohol follows a competitive inhibition mechanism. Then, three proposed kinetic models and numerical determination of parameters were applied for simulating the effect of the ethanol concentration on the kinetic reactions of transesterification of palm oil by the immobilized lipase.

3.1.1. Mechanism 1

In mechanism 1, starting with the reaction network shown in Fig. 2, one can derive many rate expressions for the various cases in which different steps are taken as rate determining. All reactions for ester production were classed into two groups: one was hydrolysis and another was esterification. Any hydrolysis reaction that produced free fatty acid included a stepwise process of hydrolysis of triacylglycerol, diacylglycerol and monoacylglycerol. The esterification reaction for fatty acid ethyl ester with excess ethanol is a synthesis reaction. The transesterification reaction therefore involves sequential execution of the hydrolysis and esterification steps, and thus requires multiple entrances and exits of reactant and product species in such a manner as to render the overall mechanism as the Ping-Pong type. Alternatively, some lipases have been reported to exhibit a rate-determining step at the point where the enzyme breaks the complex of enzyme and substrate [16]. In this mechanism, the expressions for the rates of appearance of hydrolyzed intermediates and synthesized fatty acid ethyl ester are assumed to be rate-limiting steps and the remaining steps are assumed to be in rapid equilibrium.

The appearance rates of hydrolyzed intermediates including triacylglycerol (*T*), diacylglycerol (*D*), monoacylglycerol (*M*) and glycerol (*G*) are expressed as in Eqs. (1)–(4). The appearance rates of free fatty acid (*F*), water (*W*), fatty acid ethyl ester (Es) and ethanol (Al) from the hydrolysis and esterification steps are presented in Eqs. (5)–(7).

$$\frac{d[T]}{dt} = -k_3[E\cdot T] + k_4[E\cdot D\cdot F] \quad (1)$$

$$\frac{d[D]}{dt} = k_3[E\cdot T] - k_4[E\cdot D\cdot F] - k_9[E\cdot D] + k_{10}[E\cdot M\cdot F] \quad (2)$$

$$\frac{d[M]}{dt} = k_9[E\cdot D] - k_{10}[E\cdot M\cdot F] - k_{15}[E\cdot M] + k_{16}[E\cdot G\cdot F] \quad (3)$$

$$\frac{d[G]}{dt} = k_{15}[E\cdot M] - k_{16}[E\cdot G\cdot F] \quad (4)$$

$$\frac{d[F]}{dt} = -\frac{d[W]}{dt} = k_3[E\cdot T] - k_4[E\cdot D\cdot F] + k_9[E\cdot D] - k_{10}[E\cdot M\cdot F] + k_{15}[E\cdot M] - k_{16}[E\cdot G\cdot F] - k_{19}[E\cdot F] \quad (5)$$

$$\frac{d[Es]}{dt} = k_{19}[E\cdot F][Al] \quad (6)$$

$$\frac{d[Al]}{dt} = -k_{22}[E][Al] + k_{23}[E\cdot Al] - k_{19}[E\cdot F][Al] \quad (7)$$

where *E* is the free enzyme concentration and *E*·*T*, *E*·*D*, *E*·*M*, *E*·*F*, *E*·Al, *E*·*D*·*F*, *E*·*M*·*F* and *E*·*G*·*F* are different complexes between the enzyme and the species defined above. The concentrations of the different enzymatic complexes can be expressed in terms of the free enzyme concentration by means of the following pseudoequilibrium relationships:

$$[E\cdot T] = \frac{k_1}{k_2}[E][T] \quad (8)$$

$$[E\cdot D] = \frac{k_7}{k_8}[E][D] \quad (9)$$

$$[E\cdot M] = \frac{k_{13}}{k_{14}}[E][M] \quad (10)$$

$$[E\cdot F] = \frac{k_{20}}{k_{21}}\frac{[E][F]}{[W]} \quad (11)$$

$$[E\cdot Al] = \frac{k_{22}}{k_{23}}[E][Al] \quad (12)$$

$$[E\cdot D\cdot F] = \frac{k_6}{k_5}[E\cdot F][D] = \frac{k_6k_{20}}{k_5k_{21}}\frac{[E][F]}{[W]}[D] \quad (13)$$

$$[E\cdot M\cdot F] = \frac{k_{12}}{k_{11}}[E\cdot F][M] = \frac{k_{12}k_{20}}{k_{11}k_{21}}\frac{[E][F]}{[W]}[M] \quad (14)$$

$$[E\cdot G\cdot F] = \frac{k_{18}}{k_{17}}[E\cdot F][G] = \frac{k_{18}k_{20}}{k_{17}k_{21}}\frac{[E][F]}{[W]}[G] \quad (15)$$

The total enzyme concentration (E_T) is given by

$$E_T = E + E\cdot T + E\cdot D + E\cdot M + E\cdot F + E\cdot D\cdot F 6 + E\cdot M\cdot F + E\cdot G\cdot F + E\cdot Al \quad (16)$$

By substitution of Eqs. (8)–(16) into Eqs. (1)–(7), and algebraic manipulation of the resulting equations, the following rate expressions were obtained:

$$\frac{d[T]}{dt} = (-V_{mT}[T] + V_{rT}\frac{[F][D]}{[W]})[E^*] \quad (17)$$

$$\frac{d[D]}{dt} = (V_{mT}[T] - V_{rT}\frac{[F][D]}{[W]} - V_{mD}[D] + V_{rD}\frac{[F][M]}{[W]})[E^*] \quad (18)$$

$$\frac{d[M]}{dt} = (V_{mD}[D] - V_{rD}\frac{[F][M]}{[W]} - V_{mM}[M] + V_{rM}\frac{[F][G]}{[W]})[E^*] \quad (19)$$

$$\frac{d[G]}{dt} = (V_{mM}[M] - V_{rM}\frac{[F][G]}{[W]})[E^*] \quad (20)$$

$$\frac{d[F]}{dt} = -\frac{d[W]}{dt} = (V_{mT}[T] + V_{mD}[D] + V_{mM}[M] - \frac{[F]}{[W]}(V_{rT}[D] + V_{rD}[M] + V_{rM}[G] + V_{eEs}[Al]))[E^*] \quad (21)$$

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$$\frac{d[Es]}{dt} = -\frac{d[Al]}{dt} = (V_{eEs}\frac{[F]}{[W]}[Al])[E^*] \quad (22)$$

The $[E^*]$ in Eqs. (17)–(22) is

$$[E^*] = \frac{[E_T]}{1 + K_{mT}[T] + K_{mD}[D] + K_{mM}[M] + K_{mF}([F]/[W])(1 + K_{mDF}[D] + K_{mMF}[M] + K_{mGF}[G]) + (Al/K_I)} \quad (23)$$

where V_{mT}, V_{mD} and V_{mM} are rate constants for hydrolysis of T, D and M, respectively. V_{eEs} is the rate constant for esterification of fatty acid ethyl ester. These are defined as:

$$V_{mT} = \frac{k_3 k_1}{k_2}, \quad V_{mD} = \frac{k_9 k_7}{k_8}, \quad V_{mM} = \frac{k_{15} k_{13}}{k_{14}}$$

and $V_{eEs} = \frac{k_{19} k_{20}}{k_{21}}$.

V_{rT}, V_{rD} and V_{rM} are rate constants for re-esterification of D, M and G, respectively and defined as:

$$V_{rT} = \frac{k_4 k_6 k_{20}}{k_5 k_{21}}, \quad V_{rD} = \frac{k_{10} k_{12} k_{20}}{k_{11} k_{21}} \text{ and } V_{rM} = \frac{k_{16} k_{18} k_{20}}{k_{17} k_{21}}.$$

K_{mT}, K_{mD}, K_{mM}, K_{mF}, K_{mDF}, K_{mMF} and K_{mGF} are equilibrium constants for T, D, M, F, $D{\cdot}F$, $M{\cdot}F$ and $G{\cdot}F$, respectively and defined as:

$$K_{mT} = \frac{k_1}{k_2}, \quad K_{mD} = \frac{k_7}{k_8}, \quad K_{mM} = \frac{k_{13}}{k_{14}}, \quad K_{mF} = \frac{k_{20}}{k_{21}},$$

$$K_{mDF} = \frac{k_6 k_{20}}{k_5 k_{21}}, \quad K_{mMF} = \frac{k_{12} k_{20}}{k_{11} k_{21}} \text{ and } K_{mGF} = \frac{k_{18} k_{20}}{k_{17} k_{21}}.$$

K_I is the inhibition constant for ethanol inhibition which is defined as:

$$K_I = \frac{k_{23}}{k_{22}}.$$

Eventually, in this mechanism there are 15 unknown parameters. These parameters were estimated by fitting the model equations with the experimental data obtained with various ethanol concentrations.

3.1.2. Mechanism 2

Mechanism 2 differs from mechanism 1 with respect to the step involving the decomplexation of the enzyme and intermediates after the hydrolysis reaction. It is generally accepted that when the substrate is bound to the active site, the crucial catalytic step is the hydrolysis of the ester bond and forms a mixed acyl complex of liberated fatty acid and enzyme. Once this mixed acyl complex is formed, the hydrolyzed intermediate is released [32]. Accordingly, to simplify the mechanism, the decomplexation of intermediates from the mixed acyl complex after the hydrolysis step was assumed to occur rapidly. Subsequently, the rate expressions for producing intermediates were simplified and the rate constants involving the model could be reduced. Similar rate expressions have been successfully applied in the hydrolysis reaction of triolein [14] but not yet applied in the transesterification reaction. The conceptual scheme of the overall reaction mechanism is shown in Fig. 3. The development of the rate expressions for mechanism 2 follows the same general procedure as that for mechanism 1. The resultant rate expressions for mechanism 2 are:

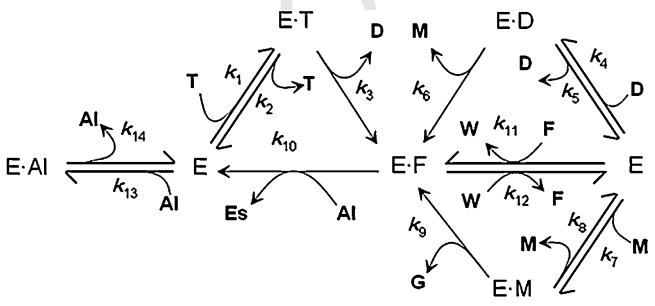

Fig. 3. Conceptual scheme of overall reaction mechanism 2.

$$\frac{d[T]}{dt} = -V_{mT}[T][E^*] \quad (24)$$

$$\frac{d[D]}{dt} = (V_{mT}[T] - V_{mD}[D])[E^*] \quad (25)$$

$$\frac{d[M]}{dt} = (V_{mD}[D] - V_{mM}[M])[E^*] \quad (26)$$

$$\frac{d[F]}{dt} = -\frac{d[W]}{dt} = (V_{mT}[T] + V_{mD}[D] + V_{mM}[M])\frac{V_{mF}[W]}{V_{mF}[W] + V_{eEs}[Al]}[E^*] + V_{rF}[F]\left(\frac{V_{mF}[W]}{V_{mF}[W] + V_{eEs}[Al]} - 1\right)[E^*] \quad (27)$$

$$\frac{d[Es]}{dt} = -\frac{d[Al]}{dt} = (V_{mT}[T] + V_{mD}[D] + V_{mM}[M] + V_{rF}[F]) \times \frac{V_{eEs}[Al]}{V_{mF}[W] + V_{eEs}[Al]}[E^*] \quad (28)$$

The $[E^*]$ in Eqs. (24)–(28) is

$$[E^*] = \frac{[E_T]}{1 + [T](K_{mT} + (V_{mT}/V_{mF}[W] + V_{eEs}[Al])) + [D]\left(K_{mD} + \frac{V_{mD}}{V_{mF}[W] + V_{eEs}[Al]}\right) + [M]\left(K_{mM} + \frac{V_{mM}}{V_{mF}[W] + V_{eEs}[Al]}\right) + \frac{V_{rF}[F]}{V_{mF}[W] + V_{eEs}[Al]} + \frac{Al}{K_I}} \quad (29)$$

where V_{mT}, V_{mD}, V_{mM} and V_{eEs} are defined as:

$$V_{mT} = \frac{k_3 k_1}{k_2 + k_3}, \quad V_{mD} = \frac{k_6 k_4}{k_5 + k_6}, \quad V_{mM} = \frac{k_9 k_7}{k_8 + k_9}$$

and $V_{eEs} = k_{10}$.

V_{mF} and V_{rF} are rate constants of hydrolysis and re-esterification, respectively, for $E{\cdot}F$. They are defined as:

$V_{mF} = k_{12}$ and $V_{rF} = k_{11}$.

K_{mT}, K_{mD}, K_{mM} and K_I are

$$K_{mT} = \frac{k_1}{k_2 + k_3}, \quad K_{mD} = \frac{k_4}{k_5 + k_6}, \quad K_{mM} = \frac{k_7}{k_8 + k_9}$$

and $K_I = \frac{k_{14}}{k_{13}}$.

In this mechanism there are 10 unknown parameters so fewer than those in mechanism 1. These parameters were estimated by fitting the model equations with the experimental data obtained with various ethanol concentrations as parameters in mechanism 1.

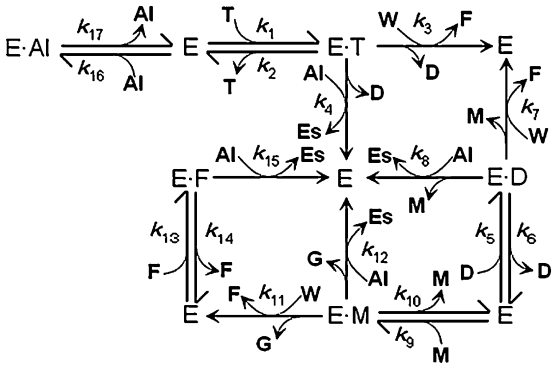

Fig. 4. Conceptual scheme of overall reaction mechanism 3.

3.1.3. Mechanism 3

Mechanism 3 differs from mechanisms 1 and 2 at the point in the kinetic reactions at which the molecule of ethanol engages. Since it was reported that it was more accurate to assume that transesterification takes place by direct alcoholysis of the triacylglycerols than two consecutive steps of hydrolysis and esterification [20], in mechanism 3 the catalytic reaction was divided into two patterns of reaction; one represents the hydrolysis step to produce free fatty acid prior to the esterification step and another represents the ethanolysis reaction to directly produce the fatty acid ethyl ester. In practice, these two steps occur simultaneously. Thus, the reaction network consists of a combination of a series of parallel reactions for each acylglycerol complex (*E·T*, *E·D* and *E·M*). The conceptual scheme of the overall reaction mechanism is shown in Fig. 4. The development of the rate expressions for mechanism 3 also follows the same general procedure as that for mechanisms 1 and 2. The resultant rate expressions for mechanism 3 are:

$$\frac{d[T]}{dt} = -(V_{mT}[W] + V_{eT}[Al])[T][E^*] \quad (30)$$

$$\frac{d[D]}{dt} = ((V_{mT}[W] + V_{eT}[Al])[T] - (V_{mD}[W] + V_{eD}[Al])[D])[E^*] \quad (31)$$

$$\frac{d[M]}{dt} = ((V_{mD}[W] + V_{eD}[Al])[D] - (V_{mM}[W] + V_{eM}[Al])[M])[E^*] \quad (32)$$

$$\frac{d[G]}{dt} = (V_{mM}[W] + V_{eM}[Al])[M][E^*] \quad (33)$$

$$\frac{d[F]}{dt} = ((V_{mT}[T] + V_{mD}[D] + V_{mM}[M])[W] - V_{eEs}[F][Al])[E^*] \quad (34)$$

$$\frac{d[W]}{dt} = -(V_{mT}[T] + V_{mD}[D] + V_{mM}[M])[W][E^*] \quad (35)$$

$$\frac{d[Es]}{dt} = -\frac{d[Al]}{dt} = (V_{eT}[T] + V_{eD}[D] + V_{eM}[M] + V_{eEs}[F])[Al][E^*] \quad (36)$$

The $[E^*]$ in Eqs. (30)–(36) is

$$[E^*] = \frac{[E_T]}{1 + K_{mT}[T] + K_{mD}[D] + K_{mM}[M] + K_{mF}[F] + (Al/K_I)} \quad (37)$$

where V_{mT}, V_{mD}, V_{mM} and V_{eEs} are defined as:

$$V_{mT} = \frac{k_3 k_1}{k_2}, \quad V_{mD} = \frac{k_7 k_5}{k_6}, \quad V_{mM} = \frac{k_{11} k_9}{k_{10}}$$

and $$V_{eEs} = \frac{k_{15} k_{13}}{k_{14}},$$

while V_{eT}, V_{eD} and V_{eM} are rate constants for ethanolysis of *T*, *D* and *M*, respectively. They are defined as:

$$V_{eT} = \frac{k_4 k_1}{k_2}, \quad V_{eD} = \frac{k_8 k_5}{k_6} \quad \text{and} \quad V_{eM} = \frac{k_{12} k_9}{k_{10}}.$$

K_{mT}, K_{mD}, K_{mM}, K_{mF} and K_I are

$$K_{mT} = \frac{k_1}{k_2}, \quad K_{mD} = \frac{k_5}{k_6}, \quad K_{mM} = \frac{k_9}{k_{10}}, \quad K_{mF} = \frac{k_{13}}{k_{14}}$$

and $$K_I = \frac{k_{17}}{k_{16}}.$$

Table 1
Parameters in the models

Parameters	Mechanism 1 ($mmol^{-1}\ h^{-1}$)	Mechanism 2 ($g^{-1}\ h^{-1}$)	Mechanism 3 ($mmol^{-1}\ h^{-1}$)
Rate constants			
V_{mT}	4.221	8.863	7.619×10^{-2}
V_{mD}	3.783	6.492	8.128×10^{-2}
V_{mM}	3.504	3.078	1.951×10^{-1}
V_{eEs}	22.731	5.594	1.383
V_{rT}	14.157	–	–
V_{rD}	11.313	–	–
V_{rM}	9.126	–	–
V_{mF}	–	5.172×10^{-2}	–
V_{rF}	–	2.281	–
V_{eT}	–	–	2.751
V_{eD}	–	–	1.176
V_{eM}	–	–	0.965
Equilibrium constants ($g\ mmol^{-1}$)			
K_{mT}	9.111×10^{-2}	9.120×10^{-2}	2.891×10^{-2}
K_{mD}	4.134×10^{-2}	8.532×10^{-2}	2.322×10^{-2}
K_{mM}	6.088×10^{-2}	7.425×10^{-2}	1.974×10^{-2}
K_{mF}	5.632×10^{-2}	–	1.121×10^{-2}
K_{mDF}	7.109×10^{-2}	–	–
K_{mMF}	5.989×10^{-2}	–	–
K_{mGF}	3.431×10^{-2}	–	–
Inhibition constant ($mmol\ g^{-1}$)			
K_I	0.924	0.894	0.882

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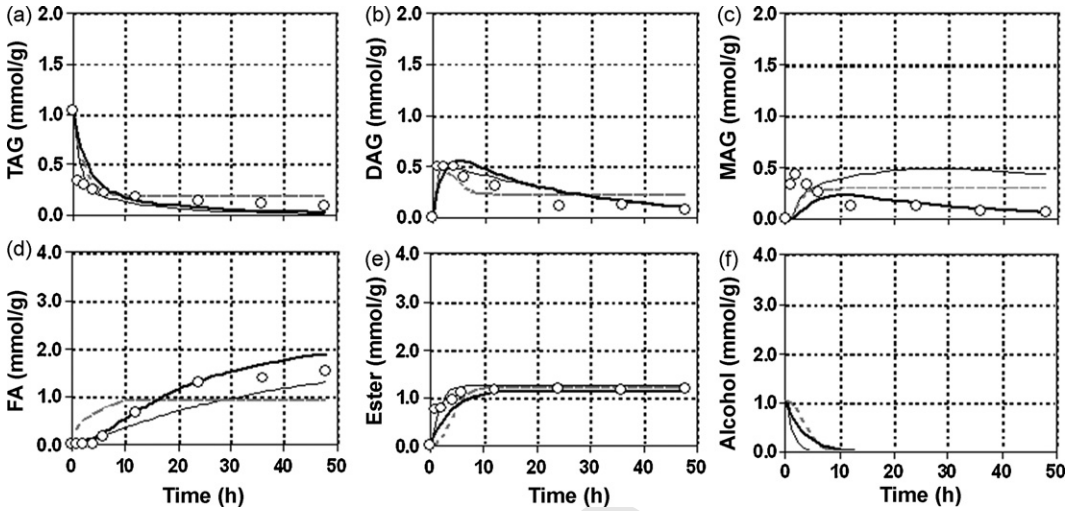

Fig. 5. Comparison between calculated (lines) and experimental results (symbols) of intermediates changes including triacylglycerol (TAG), diacylglycerol (DAG), monoacylglycerol (MAG), free fatty acid (FA) and fatty acid ethyl ester (ester) and calculated ethanol (alcohol) concentrations in the transesterification reaction with ethanol to palm oil molar ratios of 1:1. Mechanism 1:—; Mechanism 2:– – –; Mechanism 3:—.

In this mechanism there are 12 unknown parameters. These parameters were estimated by fitting the model equations with the experimental data obtained under various ethanol concentrations just as was done for the parameters in mechanisms 1 and 2.

3.2. Determination of kinetic parameters

The concentrations of triacylglycerol, diacylglycerol, monoacylglycerol, free fatty acid and fatty acid ethyl ester at different times were obtained experimentally starting with 5 g of palm oil and various ethanol amounts of 0.29, 0.58 and 0.86 g to obtain molar ratios of ethanol to palm oil of 1, 2 and 3, respectively. The amounts of enzyme and water were 0.2 g and 10% based on the palm oil weight, respectively. The nonlinear curve fitting by Simplex's method [33] was used for fitting the system of the differential equations for each mechanism into the experimental data. Simplex's method minimizes the sum of the squares of the difference between the experimental and calculated values. By fitting the above differential equations to the experimental data, the parameters involved in each model were estimated and are listed in Table 1. Based upon these determined parameters and the kinetic scheme, the concentrations of each component in the different reactions could be calculated. Comparisons between the calculated and experimental data are presented in Figs. 5–7. To evaluate the suitability of the model, the sum of squares of the residuals in each model at the

Table 2
Comparision of the sum of squares of the residuals in each model

Component	Sum of squares of the residuals		
	Mechanism 1	Mechanism 2	Mechanism 3
TAG	7.45	9.80	6.37
DAG	7.54	8.35	5.73
MAG	19.12	17.31	11.55
FA	13.09	9.03	8.14
Ester	3.84	10.20	2.59
Total sum of squares	51.03	54.69	34.39

TAG: triacylglycerol, DAG: diacylglycerol, MAG: monoacylglycerol, FA: free fatty acid, Ester: fatty acid ethyl ester.

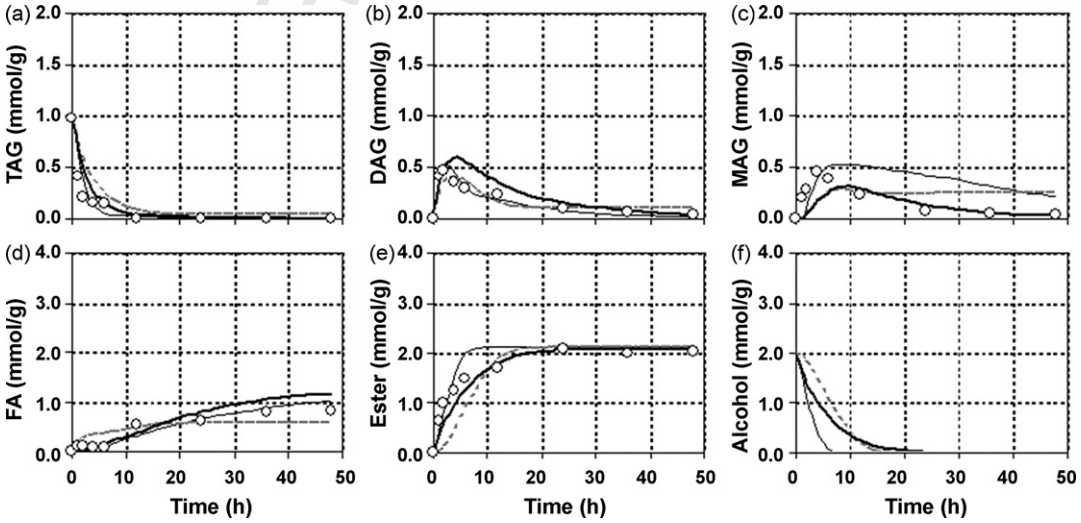

Fig. 6. Comparison between the calculated (lines) and experimental results (symbols) of intermediates changes including triacylglycerol (TAG), diacylglycerol (DAG), monoacylglycerol (MAG), free fatty acid (FA) and fatty acid ethyl ester (Ester) and calculated ethanol (Alcohol) concentration in the transesterification reaction with ethanol to palm oil molar ratio of 2:1. Mechanism 1:—; Mechanism 2:– – –; Mechanism 3:—.

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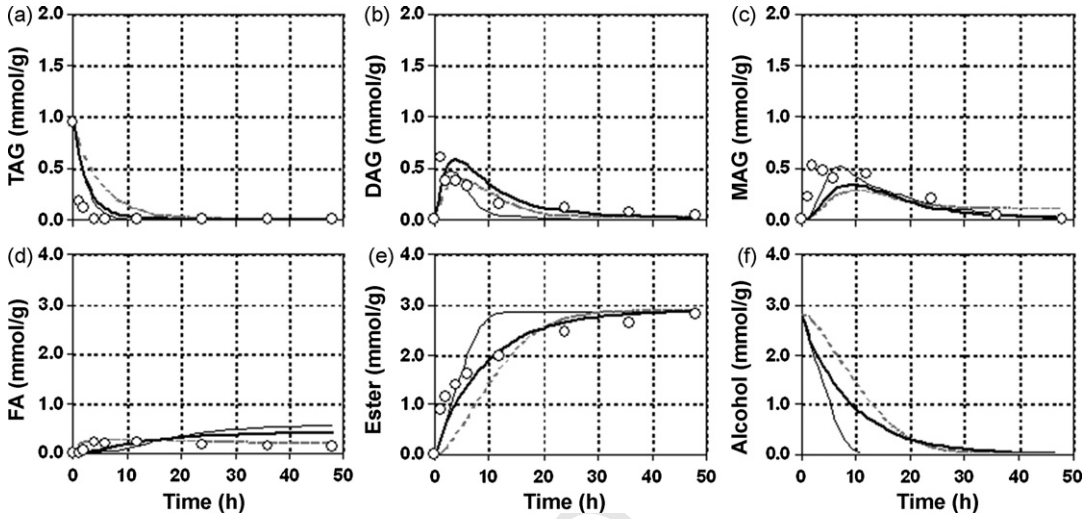

Fig. 7. Comparison between calculated (lines) and experimental results (symbols) of intermediates changes including triacylglycerol (TAG), diacylglycerol (DAG), monoacylglycerol (MAG), free fatty acid (FA) and fatty acid ethyl ester (ester) and calculated ethanol (alcohol) concentrations in the transesterification reaction with ethanol to palm oil molar ratio of 3:1. Mechanism 1:—; Mechanism 2:– – –; Mechanism 3:—.

convergence step has been calculated as presented in Table 2. The consistently lowest values of the sum of squares of the residuals for each component in mechanism 3 reveal that the mechanism 3 is the most suitable one to be applied in simulation study than the other mechanisms. A visual inspection of Figs. 5–7 also indicates that the mechanism 3 model provides a good approximation to most of the data sets. At a low ethanol concentration the simulated results of mechanisms 1 and 2 did not fit well to the experimental data especially for the monoacylglycerol concentration while at high ethanol concentrations both these mechanisms did not explain well the determined concentrations of the fatty acid ethyl ester. Thus, the concept that the two reactions of hydrolysis and transesterification occur simultaneously is more acceptable than the concept that acylglycerols are hydrolyzed and fatty acids become free prior to ethyl ester synthesis. This was confirmed by the experimental results that showed that the concentration of the measurable free fatty acid was always low during the time course of the reaction at a high initial ethanol concentration (Fig. 7) indicating that in the presence of ethanol, the fatty acid in acylglycerol was directly used in ethanolysis reaction, rather than being released as a free fatty acid by the hydrolysis reaction.

In terms of the reaction rate constants of mechanism 3 in Table 1, the rate constants of triacylglycerol, diacylglycerol and monoacylglycerol in the hydrolysis reaction ($V_{mT} = 0.07619\ mmol^{-1}\ h^{-1}$, $V_{mD} = 0.08128\ mmol^{-1}\ h^{-1}$, $V_{mM} = 0.1951\ mmol^{-1}\ h^{-1}$) were much lower than those in the ethanolysis reaction ($V_{eT} = 2.751\ mmol^{-1}\ h^{-1}$, $V_{eD} = 1.176\ mmol^{-1}\ h^{-1}$ and $V_{eM} = 0.965\ mmol^{-1}\ h^{-1}$). This indicated that in the presence of ethanol the acylglycerols were more easily converted to fatty acid ethyl ester through ethanolysis reaction rather than to free fatty acid through hydrolysis reaction. This result also supports the assumption that transesterification of triacylglycerol occurs directly by the alcoholysis reaction as has been reported in the literature [20]. However, in those studies, the behavior of the substrates and intermediates, namely acylglycerols and free fatty acids were not considered. The results presented in this paper also showed that the concentration of measurable intermediate free fatty acid was low during the time course of the reaction at high ethanol concentrations (Fig. 7). This result implied that the ethanolysis reaction is dominant over the hydrolysis reaction at a high initial ethanol concentration. On the other hand, the hydrolysis reaction might require higher concentrations of water to increase the hydrolysis reaction rate. For a further study, the estimated parameters of mechanism 3 in Table 1 were used in computer simulations to investigate the sensitivity of the model and the impact of the ethanol concentration on the transesterification reaction of palm oil by the immobilized lipase.

3.3. Sensitivity of the model

A sensitivity analysis of the parameters in the model was carried out by calculating the initial production rates of fatty acid ethyl ester and free fatty acid under condition where the value of one parameter was changed without changing any other parameter. Table 3 shows the effects of parameters on the predicted initial production rates of fatty acid ethyl ester and free fatty acid by increasing or decreasing the parameter values by 50%. The results show that the changes of V_{eT}, V_{eD} and V_{eM} produced larger differences (2–31%) on the production rates of fatty acid ethyl ester than did V_{mT}, V_{mD}, and V_{mM} (0.01–0.35%). As the values of V_{eT}, V_{eD}, V_{eM}, and V_{eEs} increased by 50% the production rates of fatty acid ethyl ester also increased 0.88–19.4%, however, the concentration of fatty

Table 3
Sensitivity of kinetic parameters on initial production rates of fatty acid ethyl ester (r_{Es}) and free fatty acid (r_F)

Initial rate	Parameter change	Deviation of initial rate with parameter change (%)											
		V_{mT}	V_{mD}	V_{mM}	V_{eEs}	V_{eT}	V_{eD}	V_{eM}	K_{mT}	K_{mD}	K_{mM}	K_{mF}	K_I
r_{Es}	+50%	+0.35	+0.24	+0.01	+0.88	+19.4	+8.95	+1.81	−0.17	−0.13	−0.05	−0.01	+20.8
	−50%	−0.35	−0.25	−0.01	−1.14	−31.4	−12.1	−2.11	+0.17	+0.13	+0.03	+0.01	34.2
r_F	+50%	+14.3	+21.2	+12.7	−14.4	+25.1	+6.22	−1.68	−0.28	−0.23	−0.05	−0.01	+38.6
	−50%	−14.9	−22.3	13.8	+18.8	−23.3	−7.08	+1.87	+0.28	0.22	+0.04	+0.01	−46.7

+, −: The values increase or decrease from the values without parameter change.

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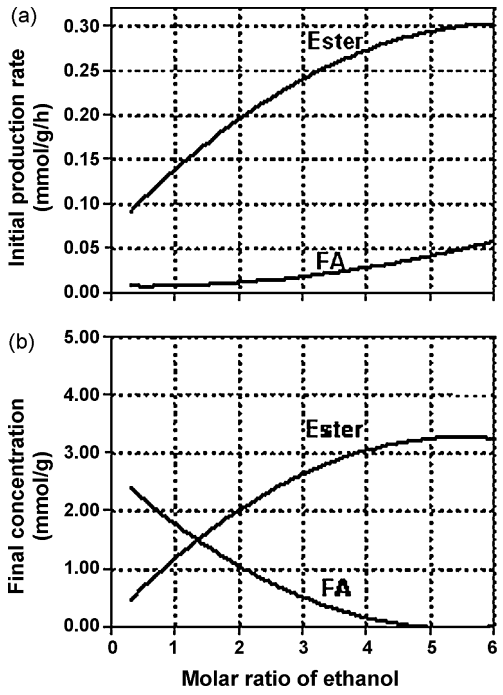

Fig. 8. Simulation results of the effects of molar ratio of ethanol on the initial production rates (a) and final concentrations (b) of fatty acid ethyl ester (ester) and free fatty acid (FA).

acid ethyl ester at equilibrium was not affected (data not shown). On the other hand, as the values of V_{mT}, V_{mD}, and V_{mM} increased by 50%, the production rates of free fatty acid increased 12–15%. This result confirmed that the parameters involved in the ethanolysis reaction (V_{eT}, V_{eD} and V_{eM}) had the most important role on the prediction of fatty acid ethyl ester production whereas the parameters involved in the hydrolysis reaction (V_{mT}, V_{mD} and V_{mM}) had the biggest effect on the prediction of free fatty acid production. In addition, the reaction rates increased when K_{mT}, K_{mD}, K_{mM} and K_{mF} were decreased. However, among the equilibrium constants, K_I produced the largest difference (20–46%) to the reaction rates. This confirmed that the model could be applied to predict the impact of the ethanol concentration on biodiesel production by the immobilized lipase.

3.4. Simulations

Because no solvent was used in this system, it was physically impossible to change the concentration of one reactant without changing that of the others. However, computer simulations allow one to independently assess the effects of hypothetical changes in the concentrations of each substrate and intermediate. Furthermore, the computer simulations also permit one to examine the dynamics and equilibria of the transesterification reaction that cannot be investigated experimentally. Beyond these limitations, the model based on mechanism 3 provides a useful description of the transesterification of palm oil with ethanol by immobilized lipase. The estimated parameters were considered to be the most representative of the palm oil data. The effects of changes in the value of the initial concentrations of alcohol were investigated in a series of simulations. The calculated results using the model of mechanism 3 are shown in Fig. 8. The initial ethanol concentration was varied to obtain molar ratios of ethanol to palm oil in the range of 0.5–6 while all other initial conditions (water 10% based on oil weight and enzyme amount 0.2 g) were held constant. Fig. 8a indicates that higher initial concentration of ethanol lead to a faster initial rate of fatty acid ethyl ester production. The result is expected from a kinetic point of view, because a higher concentration of ethanol could enhance the production rate of the fatty acid ethyl ester. Hence, much higher ethanol concentrations would be required for inhibition to take place in a solvent free system. By comparing the initial rates of fatty acid ethyl ester and free fatty acid production as shown in Fig. 8a, the initial rates of fatty acid ethyl ester production were found to be much higher than those of free fatty acid production. This indicated that ethanol is incorporated faster than is water as was also indicated by the higher values of the rate constants involving the ethanolysis reaction than those in the hydrolysis reaction (Table 1). It was also found that the production rates of free fatty acid also increased with increasing ethanol concentrations. This might be due to the thermodynamic shift from monoacylglycerol to free fatty acid when a high concentration of monoacylglycerol was obtained from the ethanolysis reaction.

In addition, the higher initial concentrations of ethanol resulted in greater amounts of ethanol being incorporated into the fatty acid of palm oil (at equilibrium) as shown in Fig. 8b. From a thermodynamic standpoint, a greater incorporation of ethanol is expected because a higher concentration of this ethanol should shift the equilibrium toward greater transesterification. However, a molar ratio of ethanol to palm oil higher than 4, resulted only a slight increase in the final concentration of fatty acid ethyl ester. Theoretically transesterification of one mole of triacylglycerol needs 3 mol of ethanol, however the yields of fatty acid ethyl ester depend on the preferred equilibrium in various conditions. The simulation results also show that increasing the initial ethanol concentration produces a lowering of the final concentration of fatty acid due to the thermodynamic equilibrium shift to transesterification. On the other hand, higher yield of free fatty acid was produced at a lower ethanol concentration.

4. Conclusions

The kinetics of a lipase-catalyzed transesterification of triacylglycerol with ethanol for biodiesel production were successfully modeled using rate expressions requiring adjustable parameters. The mechanism for transesterification by the immobilized lipase was determined by the construction of various mechanisms in the models and then fitted to the experimental data. The simulation results indicated that the two reactions of hydrolysis and ethanolysis occurred simultaneously rather than by the stepwise hydrolysis followed by esterification. Furthermore, unlike previous reports in the literature, the present model was able to account for the effects of concentrations of all chemical species participating in the transesterification reaction throughout the entire reaction especially the impact of the ethanol concentration. From a thermodynamic standpoint, a greater incorporation of ethanol is expected because a higher concentration of this acyl acceptor should shift the equilibrium towards a faster transesterification reaction. However, higher ethanol concentrations could inhibit the reaction. Hence, the model that included the inhibition by ethanol could predict the appropriate conditions for the efficient production of the fatty acid ethyl ester.

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References

[1] K. Krisnangkura, R. Simamaharnnop, Continuous transmethylation of palm oil in an organic solvent, J. Am. Oil Chem. Soc. 69 (1992) 166–169.
[2] M.A. Kalam, H.H. Masjuki, Biodiesel from palm oil—an analysis of its properties and potential, Biomass Bioenergy 23 (2002) 471–479.
[3] E. Crabbe, C. Nolasco-Hipolito, G. Kobayashi, K. Sonomoto, A. Ishizaki, Biodiesel production from crude palm oil and evaluation of butanol extraction and fuel properties, Process Biochem. 37 (2001) 65–71.
[4] Y. Shimada, Y. Watanabe, T. Samukawa, A. Sugihara, H. Noda, H. Fukuda, Y. Tominaga, Conversion of vegetable oil to biodiesel using immobilized *Candida antarctica* lipase, J. Am. Oil Chem. Soc. 76 (7) (1999) 789–793.
[5] Y. Shimada, Y. Watanabe, A. Sugihara, Y. Tominaga, Enzymatic alcoholysis for biodiesel fuel production and application of the reaction to oil processing, J. Mol. Catal. B: Enzym. 17 (2002) 133–142.
[6] M.M. Soumanou, U.T. Bornscheuer, Improvement in lipase-catalyzed synthesis of fatty acid methyl esters from sunflower oil, Enzyme Microb. Technol. 33 (2003) 97–103.
[7] W. Du, Y. Xu, D. Liu, J. Zeng, Comparative study on lipase-catalyzed transformation of soybean oil for biodiesel production with different acyl acceptors, J. Mol. Catal. B: Enzym. 30 (2004) 125–129.
[8] H. Noureddini, X. Gao, R.S. Philkana, Immobilized *Pseudomonas cepacia* lipase for biodiesel fuel production from soybean oil, Bioresource Technol. 96 (2005) 769–777.
[9] L. Li, W. Du, D. Liu, L. Wang, Z. Li, Lipase-catalyzed transesterification of rapeseed oils for biodiesel production with a novel organic solvent as the reaction medium, J. Mol. Catal. B: Enzym. 43 (2006) 58–62.
[10] R.D. Abigor, P.O. Uadia, T.A. Foglia, M.J. Haas, K.C. Jones, E. Okpefa, J.U. Obibuzor, M.E. Bafor, Lipase-catalysed production of biodiesel fuel from some Nigerian lauric oils, Biochem. Soc. Trans. 28 (6) (2000) 979–981.
[11] M.M.R. Talukder, S.M. Puah, J.C. Wu, W.J. Choi, Y. Chow, Lipase-catalyzed methanolysis of palm oil in presence and absence of organic solvent for production of biodiesel, Biocatal. Biotransform. 24 (4) (2006) 257–262.
[12] F. Taylor, M.J. Kurantz, J.C. Craig, Kinetics of continuous hydrolysis of tallow in a multi-layered flat-plate immobilized-lipase reactor, J. Am. Oil Chem. Soc. 69 (6) (1992) 591–594.
[13] P. Padmini, S.K. Rakshit, A. Baradarajan, Kinetics of enzymatic hydrolysis of rice bran oil in organic system, Enzyme Microb. Technol. 16 (1994) 432–435.
[14] H. Hermansyah, M. Kubo, N. Shibasaki-Kitakawa, T. Yonemoto, Mathematical model for stepwise hydrolysis of triolein using *Candida rugosa* lipase in biphasic oil–water system, Biochem. Eng. J. 31 (2006) 125–132.
[15] R. Lortie, M. Trani, F. Ergan, Kinetic study of the lipase-catalyzed synthesis of triolein, Biotechnol. Bioeng. 41 (1993) 1021–1026.
[16] H.R. Reyes, C.G. Hill, Kinetic modeling of interesterification reactions catalyzed by immobilized lipase, Biotechnol. Bioeng. 43 (1994) 171–182.
[17] Y. Xu, X. Du, D. Liu, Study on the kinetics of enzymatic interesterification of triglycerides for biodiesel production with methyl acetate as the acyl acceptor, J. Mol. Catal. B: Enzym. 32 (2005) 241–245.
[18] B. Cheirsilp, W. Kaewthong, A. H-Kittikun, Kinetic study of glycerolysis of palm olein for monoacylglycerol production by immobilized lipase, Biochem. Eng. J. 35 (1) (2007) 71–80.
[19] S. Al-Zuhair, Production of biodiesel by lipase-catalyzed transesterification of vegetable oils: a kinetic study, Biotechnol. Prog. 21 (5) (2005) 1442–1448.
[20] S. Al-Zuhair, F.W. Ling, L.S. Jun, Proposed kinetic mechanism of the production of biodiesel from palm oil using lipase, Process Biochem. 42 (2007) 951–960.
[21] J.W. Chen, W.T. Wu, Regeneration of immobilized *Candida antarctica* lipase for transesterification, J. Biosci. Bioeng. 95 (5) (2003) 466–469.
[22] L.C. Meher, D.V. Sagar, S.N. Naik, Technical aspects of biodiesel production by transesterification—a review, Renew. Sustain. Energy Rev. 10 (2006) 248–268.
[23] H. Yamada, Y. Sorimachi, T. Tagawa, Operation optimization of lipase-catalyzed biodiesel production, J. Chem. Eng. Jpn. 40 (7) (2007) 571–574.
[24] W. Kaewthong, A. H-Kittikun, Glycerolysis of palm olein by immobilized lipase PS in organic solvents, Enzyme Microb. Technol. 35 (2004) 218–222.
[25] R. Rosu, Y. Uozaki, Y. Iwasaki, T. Yamane, Repeated use of immobilized lipase for monoacylglycerol production by solid-phase glycerolysis of olive oil, J. Am. Oil Chem. Soc. 74 (4) (1997) 445–450.
[26] T. Tatara, T. Fujii, T. Kawase, M. Minagawa, Determination of tri-, di-, monooleins, and free oleic acid by the thin-layer chromatography-flame ionization detector system using internal standards and boric acid impregnated chromarods, Lipids 18 (1983) 732–736.
[27] Y. Shimada, K. Maruyama, S. Okazaki, M. Nakamura, A. Sugihara, Y. Tominaga, Enrichment of polyunsaturated fatty acids with *Geotrichum candidum* lipase, J. Am. Oil Chem. Soc. 71 (1994) 951–954.
[28] S.Y. Lee, J.S. Rhee, Production and partial purification of a lipase from *Pseudomonas putida* 3SK, Enzyme Microb. Technol. 15 (1993) 617–623.
[29] J.M.A. Danby, Computer Modeling, Willmann-Bell Inc., Richmond Va, 1997.
[30] J.P. Chen, H.Y. Wang, Improved properties of bilirubin oxidase by entrapment in alginate-silicate sol–gel matrix, Biotech. Technol. 12 (11) (1998) 851–853.
[31] M.D. Romero, L. Calvo, C. Alba, A. Daneshfar, A kinetic study of isoamyl acetate synthesis by immobilized lipase-catalyzed acetylation in *n*-hexane, J. Biotechnol. 127 (2007) 269–277.
[32] F.X. Malcata, H.R. Reyes, H.S. Garcia, C.G. Hill Jr., C.H. Amundson, Kinetics and mechanisms of reactions catalyzed by immobilized lipases, Enzyme Microb. Technol. 14 (1992) 426–446.
[33] J.A. Nelder, R. Mead, A simplex method for function minimization, Comput. J. 7 (1964) 308–313.

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Synthesis of Fatty Acid Alkyl Esters from Palm Olein Using Immobilized Lipase

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Four commercial lipases from *Pseudomonas* sp. (lipase PS), *Pseudomonas fluorescens* (lipase AK), *Candida rugosa* (lipase AY) and *Rhizopus delemar* (lipase D) were screened for production of fatty acid ethyl esters (FAEE) from palm olein. Lipase PS was the only suitable enzyme for synthesis of FAEE from palm oil. Accurel EP-200 was used as support to immobilize lipase PS. The optimum condition for immobilization was enzyme concentration of 50 U/ml and immobilization time for 30 min. The activity of immobilized lipase PS was 0.258 U/mg and immobilized yield was 83.79%. The optimum condition for FAEE production from 5 g of palm olein was found to be 50U of immobilized lipase PS, 0.5 ml of water addition and at temperature 45°C. The optimal molar ratios of palm olein to ethanol and methanol for production of FAEE and fatty acid methyl esters (FAME), respectively, were the same at 1:3. Under these conditions, the yields of 61% FAEE and 60% FAME were obtained at 6 h. Moreover, three-step addition of alcohol has improved yields of FAEE and FAME up to 76% and 75%, respectively.

Keywords: fatty acid alkyl ester, immobilized lipase, palm oil, transesterification

Introduction

Biodiesel-fatty acid alkyl esters of natural fats and oils have attracted much research interest. They are renewable while the availability of petroleum chemicals is finite. Fatty acid alkyl esters are produced by transesterification of triglycerides with alcohols preferentially methanol and ethanol. Biodiesel from palm oil can be domestically produced, offering the possibility of reducing petroleum imports. Industrial

practice for production of biodiesel from palm oil is typically produced by alkaline or acid catalyzed transesterification (Krisnangkura and Simamaharnnop, 1992; Crabbe *et al.*, 2001; Kalam and Masjuki, 2002). However, the undesired side reaction, saponification, occurs and an extra separation step to remove the homogeneous catalysis is required, thus reflecting the high cost of production.

Recently, utilization of lipase in the production of fatty acid alkyl esters has been shown to be effective. In the transesterification of soybean oil, 80-90% conversion of esters was obtained using *Rhizopus oryzae* lipase solution (Kaieda *et al.*, 1999). Lipase-catalyzed synthesis reaction selectivity is high and lipase can be immobilized in the support material, which will allow for its recovery and reuse. Transesterifications of various plant oils using immobilizied lipases have been studied. Dossat *et al.* (2002) and Soumanou and Bornscheuer (2003) both synthesized methyl esters from sunflower oil in solvent-free system using immobilized *Rhizomucor miehei* with conversion of 60% and 80%, respectively. Du *et al.* (2004) synthesized methyl esters from soybean oil using immobilized *Candida antarctica* lipase B with conversion higher than 80% by stepwise additions of methanol. Noureddini *et al.* (2005) synthesized 67% of methyl ester and 65% of ethyl ester from soybean oil using immobilized *Pseudomonas cepacia* lipase. Li *et al.* (2006) reported transesterification of rapeseed oil using combination of immobilizied *Thermomyces lanuginose* lipase and *Candida antarctica* lipase with 95% conversion of esters. Shimada *et al.* (1999) and Watanabe *et al.* (2000) used immobilized *Candida antarctica* lipase (Novozym 435) for the conversion of vegetable oil to biodiesel. Results showed incomplete methanolysis of vegetable oil which was attributed to the inactivation of the enzyme. Stepwise addition of methanol prevented this inactivation and conversions in excess of 98% were obtained.

Several researchers have investigated biodiesel production from palm oil using immobilized lipases. Abigor *et al.* (2000) studied the lipase-catalyzed transesterification of palm kernel oil with different alcohols using lipase PS-30. Talukder *et al.* (2006) used *Candida antarctica* lipase to produce methyl esters from palm oil in presence and absence of organic solvent. However, the bottleneck of lipase-catalyzed biodiesel production is the high price of the enzyme. Since the price of *Candida antarctica* lipase is relatively expensive and far from practical, the study of other low-cost lipases for biodiesel production from palm oil is needed. Kaieda *et al.* (2001) reported that lipases from a number of microorganisms are able to catalyze methanolysis with appropriate water and methanol contents in the reaction mixture. Lipase that is relatively resistant to methanol, such as that from *Pseudomonas cepacia,* will be advantageous in low water content, which is desirable to attain a high reaction rate of transesterification.

The purpose of our research was to employ enzymatic method in industrial production of biodiesel from palm oil. The first step in present study was to investigate synthesis of fatty acid ethyl esters (FAEE) from palm oil by various lipases in solvent-free system. For industrial application, immobilization of lipase was optimized. Optimal reaction parameters for synthesis of fatty acid alkyl esters from palm oil were also determined: enzyme loading, water addition, temperature, molar ratio of substrates and three-step addition of alcohols.

Materials and Methods

Materials Lipase from *Pseudomonas* sp. (lipase PS), *Pseudomonas fluorescens*

(lipase AK), *Candida rugosa* (lipase AY) and *Rhizopus delemar* (lipase D) were gifts from Amano Pharmaceutical Co. Ltd., Nagoya, Japan. The supports were hydrophobic polypropylene powder EP200 (Accurel) was a gift from Akzo Nobel (Obernburg, Germany). Palm olein was purchased from Morakot Industry Co. Ltd., Thailand. All other chemicals were also obtained from commercial sources.

Immobilization The supports in powdered form (0.2 g) was treated with ethanol before added to 20 ml lipase solution and stirred with a magnetic bar at 100 rpm for 1 h. Afterwards, the suspension was filtered through a Buchner funnel. The immobilized enzyme was washed on the filter paper with another 5.0 ml of 0.1M phosphate buffer pH 7.0 and dried in a vacuum desiccator for 12 h. For this immobilization study, the immobilized yield was calculated using the following formula:

$$\text{Immobilized yield (\%)} = \frac{C_0V_0 - C_fV_f}{C_0V_0} \times 100$$

where C_0 is the initial activity of lipase solution (U ml^{-1}); V_0 is the initial volume of lipase solution (ml); C_f is the lipase activity of the filtrate (U ml^{-1}); and V_f is the filtrate volume (ml).

Transesterification Each lipase was investigated for its ability to catalyze transesterification of palm oil. The substrate mixture consisted of 5 g palm oil, 3 molar equivalents of ethanol to palm oil and 1 ml 0.1 M phosphate buffer pH 7.0 containing 30 U of enzyme. The reaction was maintained at 45 ◦C.

Analysis The course of transesterification was monitored by intermittent sampling (150 mg) followed by chloroform extraction. The extract was analyzed for FAEE, fatty acid methyl esters (FAME), triacylglycerol (TAG), 1,3-diacylglycerol (1,3-DAG), 1,2-diacylglycerol (1,2-DAG), MAG and free fatty acid (FFA) using a thin-layer chromatography/flame ionization detection (TLC/FID) (IATROSCAN MK5, Iatron Laboratories Inc., Tokyo) (Rosu *et al.*, 1997). In this paper, the percentage of peak area was assumed as percentage content of the corresponding compound. Hydrolytic activity of the lipase was assayed by a modified cupric acetate method (Lee and Rhee, 1993). One unit of hydrolytic activity is defined as the amount of the enzyme that liberates 1μmole equivalent of palmitic acid from palm olein in 1 min at 30°C.

All the data presented in this paper at various conditions were the mean of three experiments. Appropriate tests of significance, analysis of variance (ANOVA) and confident difference at 5% level were used in the data evaluations.

Results

Screening of lipase In a typical reaction, the same amount of 30 U hydrolytic activity of each enzyme was evaluated in transesterification of 5 g palm oil with 3 molar equivalents of ethanol. Four commercial lipases were screened for their ability to produce FAEE from palm olein. The screening results for the tested lipases are presented in Table 1. Reaction products are presented as % FAEE content in the reaction mixture. The formation of free fatty acids is also included in this table since the presence of water in the reaction medium naturally promotes the competing hydrolysis reaction. Among the tested lipases, lipase PS from *Pseudomonas* sp. showed the highest activity toward the transesterification of palm oil with ethanol. Other lipases showed very little activity toward the transesterification reaction. After 6 h of reaction with lipase PS, the product contained 57% FAEE, 6% of fatty acids, 24% of monoglycerides, 13% of diglyceride, and 0% of triglycerides.

Optimal conditions for enzyme immobilization The effect of the enzyme concentration on immobilization of lipase

PS with Accurel was investigated as shown in Table 2. The activity of the immobilized lipase increased with increasing enzyme concentration. However, at enzyme concentration higher than 50 U ml^{-1} all lipase molecules could not load onto Accurel resulted in low immobilization yield. When immobilized yield was considered, the concentration of enzyme with 50 U ml^{-1}, was suitable for lipase PS immobilization on Accurel.

The effect of time on immobilization of lipase PS on Accurel was studied. The immobilized lipase activity increased while the immobilized yield decreased when the immobilization time increased (Table 3). Since Accurel is a hydrophobic polymer, a longer immobilization time was, more floatation and separation of Accurel from lipase solution occurred and resulting low immobilized yield. The immobilization time of 15 min gave highest immobilized yield but much lower immobilized lipase activity compared to that of immobilization time of 30 min. Thus, the immobilization time of 30 min, which gave high immobilized yield and immobilized activity, was sufficient to immobilize lipase PS on Accurel.

FAEE production from palm oil by immobilized lipase PS The immobilized lipase PS produced under the optimal immobilization conditions established in this study was tested for FAEE production and compared with free lipase. Fig. 1 shows time course of FAEE production by immobilized and free lipase PS. There was no significant difference in FAEE production by immobilized and free lipase. Both free and immobilized lipase PS gave a maximum FAEE yield of 60% at 6 h.

Effect of immobilized lipase PS loading The effect of immobilized lipase PS loading (U of immobilized lipase per 5 g of palm oil) on FAEE production from palm oil was presented in Fig. 2. The FAEE yield was enhanced by increasing the amount of immobilized lipase and it was highest at 50 U. Therefore, immobilized lipase PS at 50 U was used for transesterification of 5 g of palm oil.

Effect of water addition The effect of water addition on FAEE production was determined as shown in Fig. 3. The results show that increasing water addition led in higher conversion rate. However, the effect of water addition has less pronounced on FAEE yield. And there was no significant difference on FAEE production profile at the water addition beyond 0.50 ml.

Effect of temperature The effect of temperature on FAEE production was investigated. Fig. 4 shows slight changes in the transesterification activity of the immobilized lipase PS with variations in temperature. FAEE yield increased with increasing the temperature. Since there was no significant difference between transesterification activity of the immobilized lipase PS at 45 and 55°C, lower temperature at 45°C might be suitable from the economical point of view.

Effect of molar ratio of palm oil to alcohol Experiments were performed to optimize the amount of fatty acid alkyl ester production by varying the alcohol concentration. Optimum alcohol requirements were determined for both ethanol and methanol as shown in Fig. 5. The amount of alcohol added was varied from 1 to 3 molar equivalents based on the moles of palm oil. An increase in the number of moles of alcohol with respect to the palm oil resulted in an increase in the production rates and yields of FAEE and FAME as shown in Fig. 5. The optimum alcohol concentration was determined at 3 molar ratio of alcohol to palm oil for ethanol and methanol where about 61% of FAEE and 60% of FAME were formed, respectively at 6 h.

Stepwise alcoholysis of palm oil It is widely known that some enzymes are deactivated when exposed to high

concentrations of alcohol. Therefore, to avoid lipase inactivation by ethanol and methanol a method of adding stepwise was applied. The results are shown in Fig. 6. When alcohol was added to the reaction mixture, the FAEE and FAME yields increased. The FAEE and FAME contents reached to 76 and 75%, respectively at 5 h with stepwise additions of alcohols. It is remarkable that the stepwise addition of alcohol exhibits the highest conversions of palm oil to biodiesel. Since alcohols were maintained at low concentration in the reaction mixture during stepwise addition of alcohols, the effect of deactivation by alcohols was softened and the reusability of immobilized lipase PS could be improved.

Discussion and Conclusion

In biodiesel production, the preliminary screening of lipases was carried out with randomly chosen commercial available lipases. Lipase from *Pseudomonas* sp. was the most promising one for the transesterification of palm oil in this study (Table 1) as it was also reported in the transesterification of soybean oil (Kaieda *et al.*, 2001; Noureddini *et al.*, 2005) and *Jatropha* oil (Shah and Gupta, 2007). It is likely that lipase from *Pseudomonas* sp. has much higher methanol resistance than those from the others (Kaieda *et al.*, 2001), this makes it more attractive for use as an enzyme in alcoholysis reaction of palm oil.

By immobilizing the enzyme on support material, it is possible to reuse enzyme and reduce the costs of the enzyme in industrial application. In this study, selected lipase PS was immobilized on Accurel, hydrophobic polypropylene powder. The immobilization mechanism of lipase on Accurel was simple adsorption, whereby the enzyme adheres to the surface of the support particles by Van der Waals forces of attraction (Murray *et al.*, 1997). Many lipases display interfacial activation phenomena in the presence of a hydrophobic surface. The activation has been related to a conformational change of the lipase that exposes to the hydrophobic substrate a wide hydrophobic surface surrounding the catalytic site (Brzozowski *et al.*, 1991). For the same reason, lipases are strongly adsorbed by hydrophobic surfaces. Hence, lipase immobilization by adsorption may not only improve the stability and ease of product separation, but also enzyme activity.

The effects of enzyme concentration and immobilization time on immobilized yield and immobilized activity were investigated. At high enzyme concentration, immobilized lipase displayed high activity (Table 2). This could be explained that increasing the concentration of enzyme in solution increases the driving force for adsorption. The immobilization time of 30 min was enough for enzyme adsorption on Accurel (Table 3). According to Montero *et al.* (1993), *Candida rugosa* lipase was also rapidly adsorbed on Accurel and more than 60% of the activity disappeared from enzyme solution after 1 min of incubation. It was found that the FAEE production using immobilized lipase PS performed the same as free lipase PS under the same condition (Fig. 1). This result suggests that the lipase is being adsorbed onto Accurel in such a way as not to obscure the active site, resulting in remain of enzyme activity. The result also shows that mass transfer limitation in Accurel porous supports could be neglected as it was reported for other porous supports (Chen and Wang, 1998; Romero *et al.*, 2007). Salis *et al.* (2003) reported that the internal diffusion has seldom been considered in much biocatalysis work in organic media, since the reactions are usually not very fast. Shah and Gupta (2007) indicated that the immobilized *Pseudomonas cepacia* lipase on celite gave higher yield of biodiesel from *Jatropha* oil than free lipase. This is presumed to be because of larger surface area of the

biocatalyst preparation in the immobilized form. Therefore, the immobilized enzymes give better catalytic performance in non-aqueous media. However, this property also depends on type of the support. In this study, there was no significant difference in activities of free lipase and immobilized lipase on Accurel.

Experiments were performed to determine the effect of immobilized lipase PS loading on the extent of the transesterification reaction. As the amount of immobilized lipase was increased there was a sudden surge in the formation of FAEE (Fig. 2). Similar trends were observed for the transesterification of various oils (Kaieda *et al.*, 1999; Noureddini *et al.*, 2005; Li *et al.*, 2006; Shah and Gupta, 2007). Then, the effect of water addition was investigated (Fig. 3). Lipase activity generally depends on the available interfacial area. With the increased addition of water, the amount of water available for oil to form oil–water droplets increases, thereby, increasing the available interfacial area. However, since lipases usually catalyze hydrolysis in aqueous media, excess water may also stimulate the competing hydrolysis reaction. The optimum water content is a compromise between minimizing hydrolysis and maximizing enzyme activity for the transesterification reaction (Noureddini *et al.*, 2005). In this study, 0.5 ml of water addition was thought to be the most balanced amount for hydrolysis and enzyme activity in transesterification of 5 g palm oil using 50 U of immobilized lipase PS.

The effect of temperature on FAEE production was studied over the temperature range of 30–55°C (Fig. 4). The temperature effect is related to the enzyme activity and stability. Increasing temperature from 30 to 45°C, FAEE production increased from 52 to 61%. At higher temperature 55 °C, there was no significant further increase in FAEE production compared to the temperature at 45°C. Therefore, the temperature of 45°C was chosen for transesterification of palm oil from the economical point of view. Most of lipase-catalyzed biodiesel productions have also been tested at 45°C (Nelson *et al.*, 1996).

The alcohol concentrations for FAEE and FAME production were optimized (Fig. 5). At the same molar ratio of alcohol, the production rate and yield of FAME were higher than that of FAEE. Similar results were observed by Salis *et al.* (2005). Although methanol is easily available as an absolute alcohol, it is involved in enzyme inactivation processes (Meher *et al.*, 2006). The formation of FAEE is environmentally attractive because unlike methanol, ethanol is produced from renewable resources. Also, ethanol has better solvent properties than methanol for solubility of oil (Shimada *et al.*, 2002). When 2 or 3 molar equivalents of alcohols were added to the reaction mixture, the yields of esters were 52 and 60% for FAEE and 54 and 61% for FAME, although theoretically it should have reached 66 and 100%. This result indicates that the lipase was probably inactivated when the alcohol content is high. Chen and Wu (2003) reported that when the lower alcohol in the mixture was high, it caused deactivation of the enzyme due to the immiscibility between triglycerides and alcohol. In order to overcome these drawbacks, stepwise additions of alcohols to the reaction mixture have been proposed (Shimada *et al.*, 1999; Watanabe *et al.*, 2000; Shimada, *et al.*, 2002; Soumanou and Bornscheuer, 2003). Stepwise addition of alcohols was applied in transesterification of palm oil to reduce the negative effect of alcohols on lipase activity. The results show that the yield of FAEE and FAME increased significantly from 61 to 76% and 60 to 75%, respectively using stepwise addition of alcohols.

The findings of this study have shown that lipases from *Pseudomonas* sp.

(lipase PS) immobilized on Accurel are able to catalyze transesterification of palm oil with appropriated ethanol and methanol for FAEE and FAME production, respectively. However, further study on the stability and reusability of immobilized lipase is necessary.

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References

1. Abigor, R.D., Uadia, P.O., Foglia, T.A., Haas, M.J., Jones, K.C., Okpefa, E., Obibuzor, J.U. and Bafor, M.E. (2000) Lipase-catalysed production of biodiesel fuel from some Nigerian lauric oils. Biochem. Soc. Transac. 28(6), 979–981.
2. Brzozowski, A.M., Derewenda, U., Derewenda, Z.S., Dodson, G.G., Lawson, D.M., Turkenburg, J.P., Björkling, F., Huge-Jensen, B., Patkar, S.A. and Thim, L. (1991) A model for interfacial activation in lipases from the structure of a fungal lipase-inhibitor complex. Nature. 351, 491-494.
3. Chen, J.P. and Wang, H.Y. (1998) Improved properties of bilirubin oxidase by entrapment in alginate-silicate sol-gel matrix. Biotech. Technol. 12(11), 851-853.
4. Chen, J.W and Wu, W.T. (2003) Regeneration of immobilized *Candida antarctica* lipase for transesterification. J. Biosci. Bioeng. 95(5), 466-469.
5. Crabbe, E., Nolasco-Hipolito, C., Kobayashi, G., Sonomoto, K., and Ishizaki, A. (2001) Biodiesel production from crude palm oil and evaluation of butanol extraction and fuel properties. Process Biochem. 37, 65–71.
6. Dossat, V., Combes, D. and Marty, A. (2002) Lipase-catalysed transesterification of high oleic sunflower oil. Enzyme Microb. Technol. 30, 90-94.
7. Du, W., Xu, Y., Liu, D. and Zeng, J. (2004) Comparative study on lipase-catalyzed transformation of soybean oil for biodiesel production with different acyl acceptors. J. Mol. Catal. B: Enzym. 30, 125-129.
8. Kaieda, M., Samukawa, T., Kondo, A. and Fukuda, H. (2001) Effect of methanol and water contents on production of biodiesel fuel from plant oil catalyzed by various lipases in a solvent-free system. J. Biosci. Bioeng. 91(1), 12-15.
9. Kaieda, M., Samukawa, T., Matsumoto, T., Ban, K., Kondo, A., Shimada, Y., Noda, H., Nomoto, F., Ohtsuka, K., Izumoto, E. and Fukuda, H. (1999) Biodiesel fuel production from plant oil catalyzed by *Rhizopus oryzae* lipase in a water-containing system without an organic solvent. J. Biosci. Bioeng. 88(6), 627-631.
10. Kalam, M.A. and Masjuki, H.H. (2002) Biodiesel from palm oil—an analysis of its properties and potential. Biomass and Bioenergy. 23, 471 – 479.
11. Krisnangkura, K. and Simamaharnnop, R. (1992) Continuous transmethylation of palm oil in an organic solvent. J. Am. Oil Chem. Soc. 69, 166-169.
12. Lee, S.Y. and Rhee, J.S. (1993) Production and partial purification of a lipase from *Pseudomonas putida* 3SK. Enzyme Microb. Technol. 15, 617–623.
13. Li, L., Du, W., Liu, D., Wang, L. and Li, Z. (2006) Lipase-catalyzed transesterification of rapeseed oils for

biodiesel production with a novel organic solvent as the reaction medium. J. Mol. Catal. B: Enzym. 43, 58-62.
14. Meher, L.C., Vidya Sagar, D. and Naik, S.N. (2006) Technical aspects of biodiesel production by transesterification-a review. Renewable and Sustainable Energy Reviews. 10, 248–268.
15. Montero, S., Blanco, A., Virto, M.D., Landeta, L.C., Agud, I. and Solozabal, R. (1993) Immobilization of *Candida rugosa* lipase and some properties of the immobilized enzyme. Enzyme Microb. Technol. 15(3), 239–47.
16. Murray, M., Rooney, D., Van Neikerk, M., Montenegro, A. and Weatherley, L. R. (1997) Immobilization of lipase onto lipophilic polymer particles and application to oil hydrolysis. Process Biochem. 32(6), 479-486.
17. Nelson, L.A., Foglia, T.A. and Marmer, W.N. (1996) Lipase-catalyzed production of biodiesel. J. Am. Oil Chem. Soc. 73(8), 1191-1195.
18. Noureddini, H., Gao, X. and Philkana, R.S. 2005. Immobilized *Pseudomonas cepacia* lipase for biodiesel fuel production from soybean oil. Bioresour. Technol. 96: 769–777.
19. Salis, A., Pinna, M., Monduzzi, M. and Solinas, V. (2005) Biodiesel production from triolein and short chain alcohols through biocatalysis. J. Biotech. 119, 291–299.
20. Salis, A., Sanjust, E., Solinas, V. and Monduzzi, M. (2003) Characterisation of Accurel MP1004 polypropylene powder and its use as a support for lipase immobilization. J. Mol. Catal. B: Enzym. 24–25, 75–82.
21. Shah, S. and Gupta, M.N. (2007) Lipase catalyzed preparation of biodiesel from *Jatropha* oil in a solvent free system. Process Biochem. 42, 409–414.
22. Shimada, Y., Watanabe, Y., Samukawa, T., Sugihara, A., Noda, H., Fukuda, H. and Tominaga, Y. (1999) Conversion of vegetable oil to biodiesel using immobilized *Candida antarctica* lipase. J. Am. Oil Chem. Soc. 76 (7), 789–793.
23. Shimada, Y.,Watanabe, Y., Sugihara, A. and Tominaga, Y. (2002) Enzymatic alcoholysis for biodiesel fuel production and application of the reaction to oil processing. J. Mol. Catal. B: Enzym. 17, 133–142.
24. Soumanou, M.M. and Bornscheuer, U.T. (2003) Improvement in lipase-catalyzed synthesis of fatty acid methyl esters from sunflower oil. Enzyme Microb. Technol. 33, 97-103.
25. Talukder, R., Mahabubur, Md., Sze, M.P., Jin, C.W., Choi, J.W. and Yvonne, C. (2006) Lipase-catalyzed methanolysis of palm oil in presence and absence of organic solvent for production of biodiesel. Biocatalysis and Biotransformation. 24(4), 257-262.
26. Romero, M.D., Calvo, L., Alba, C. and Daneshfar, A. (2007) A kinetic study of isoamyl acetate synthesis by immobilized lipase-catalyzed acetylation in *n*-hexane. J. Biotech. 127, 269–277.
27. Rosu, R., Vozaki, Y., Iwasaki, Y. and Yamane, T. (1997) Repeated use of immobilized lipase for monoacylglycerol production by solid-phase glycerolysis of olive oil. J. Am. Oil Chem. Soc. 74(4), 445–450.
28. Watanabe, Y., Shimada, Y., Sugihara, A., Noda, H., Fukuda, H. and Tominaga, Y. (2000) Continuous production of biodiesel fuel from vegetable oil using immobilized *Candida antarctica* lipase. J. Am. Oil Chem. Soc.77 (4), 355–360.

Table 1
FAEE production using commercial lipases

Lipase	TAG (%)	FFA (%)	FAEE (%)
PS	0.68±0.63[c]	6.69±1.27[a]	56.6±2.56[a]
AK	92.5±0.95[a]	0.34±0.13[d]	4.22±0.63[b]
AY	91.0±0.57[b]	1.98±0.38[b]	1.74±0.42[c]
D	93.4±0.23[a]	0.76±0.12[c]	1.46±0.16[c]

Mean ± S.D. (n = 3).
Different letter in each column means statistically significant differences ($p<0.05$).

Table 2
Effect of enzyme loading on immobilization of lipase PS with Accurel

Enzyme concentration (U ml^{-1})	Immobilized yield (%)	Immobilized lipase activity (U mg^{-1})
5	46.56±5.04[d]	0.045±0.015[d]
30	58.44±1.68[c]	0.082±0.002[c]
50	83.79±2.27[a]	0.258±0.011[b]
100	71.86±1.51[b]	0.649±0.003[a]

Mean ± S.D. (n = 3).
Different letter in each column means statistically significant differences ($p<0.05$).

Table 3
Effect of time on immobilization of lipase PS with Accurel

Immobilization time (min)	Immobilized yield (%)	Immobilized lipase activity (U mg^{-1})
15	91.15±0.72[a]	0.101±0.003[d]
30	83.79±2.27[b]	0.258±0.011[c]
60	81.24±1.04[b]	0.285±0.010[b]
90	73.93±0.80[c]	0.350±0.002[a]

Mean ± S.D. (n = 3).
Different letter in each column means statistically significant differences ($p<0.05$).

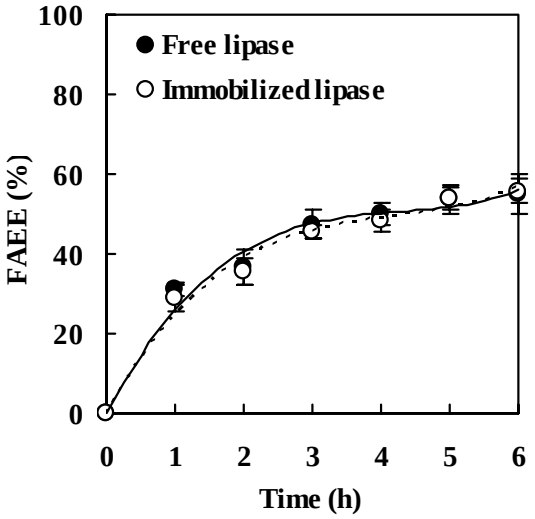

Fig. 1 Time course of FAEE production by immobilized and free lipase PS. Reaction condition; 5 g of palm oil; molar ratio of palm oil to ethanol of 1:3; 30 U of immobilized and free lipase PS; 1.00 ml of water addition and at 45°C.

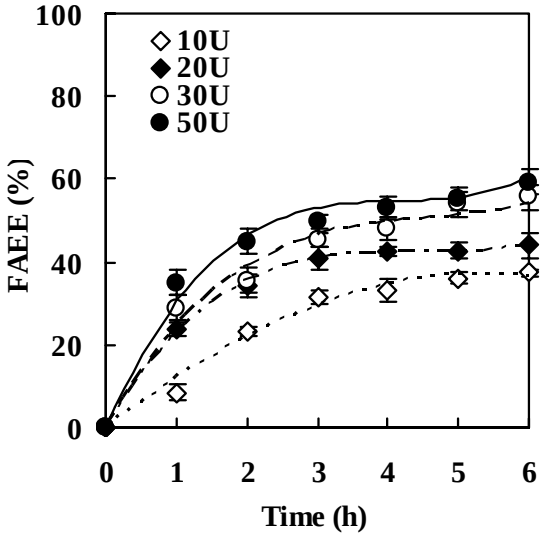

Fig. 2 Effect of immobilized lipase PS loading on FAEE production from palm oil. Reaction condition; 5 g of palm oil; molar ratio of palm oil to ethanol of 1:3; 1.00 ml of water addition and at 45°C.

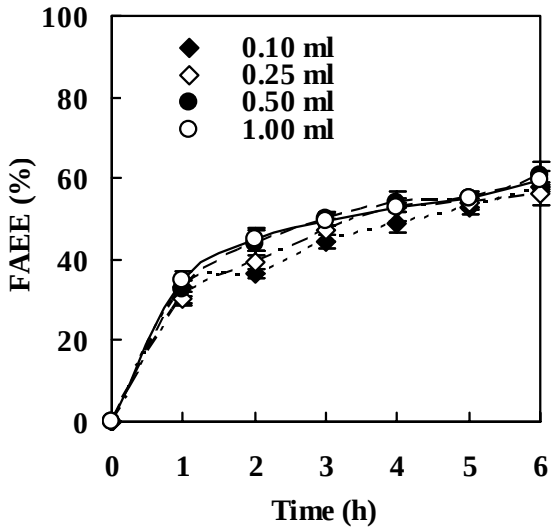

Fig. 3 Effect of water addition on FAEE production from palm oil. Reaction condition; 5 g of palm oil; 50 U of immobilized lipase PS; molar ratio of palm oil to ethanol of 1:3; and at 45°C.

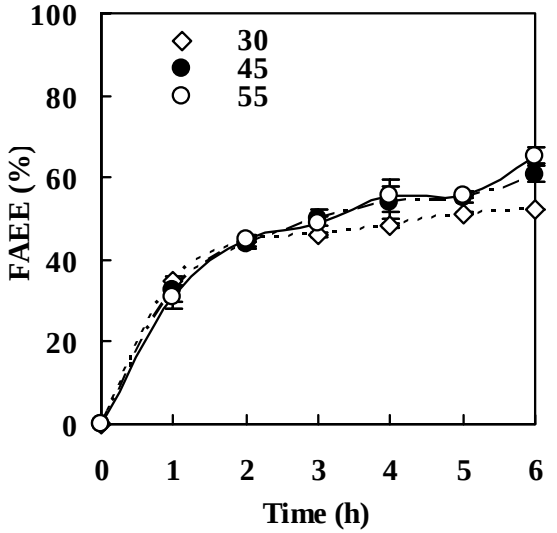

Fig. 4 Effect of temperature on FAEE production from palm oil. Reaction condition; 5 g of palm oil; 50 U of immobilized lipase PS; 0.50 ml of water addition; and molar ratio of palm oil to ethanol of 1:3.

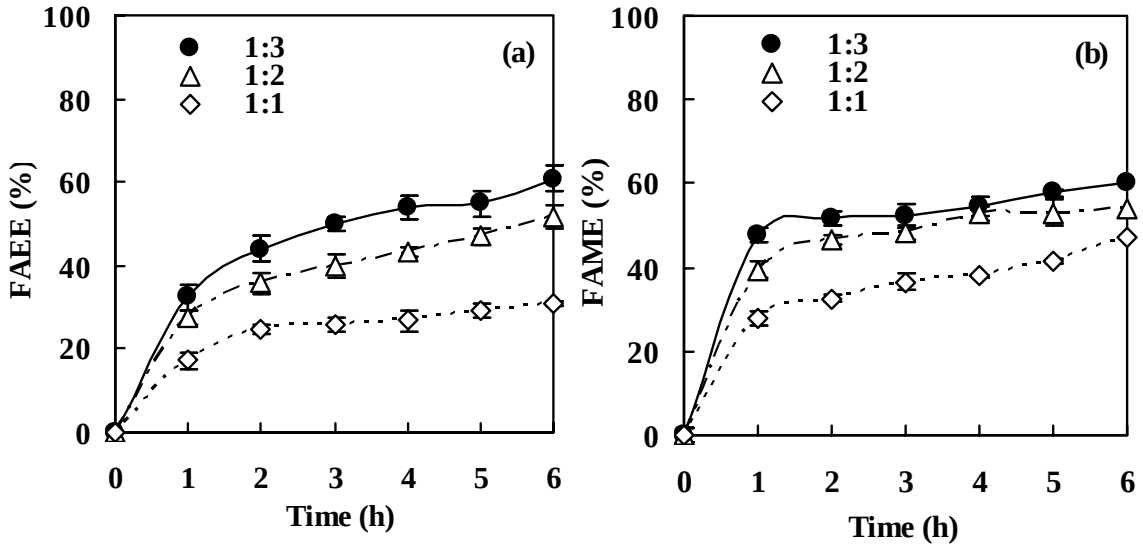

Fig. 5 Effect of molar ratio of ethanol on FAEE production (a) and molar ratio of methanol on FAME production (b). Reaction condition; 5 g of palm oil; 50 U of immobilized lipase PS; 0.50 ml of water addition; and at 45°C.

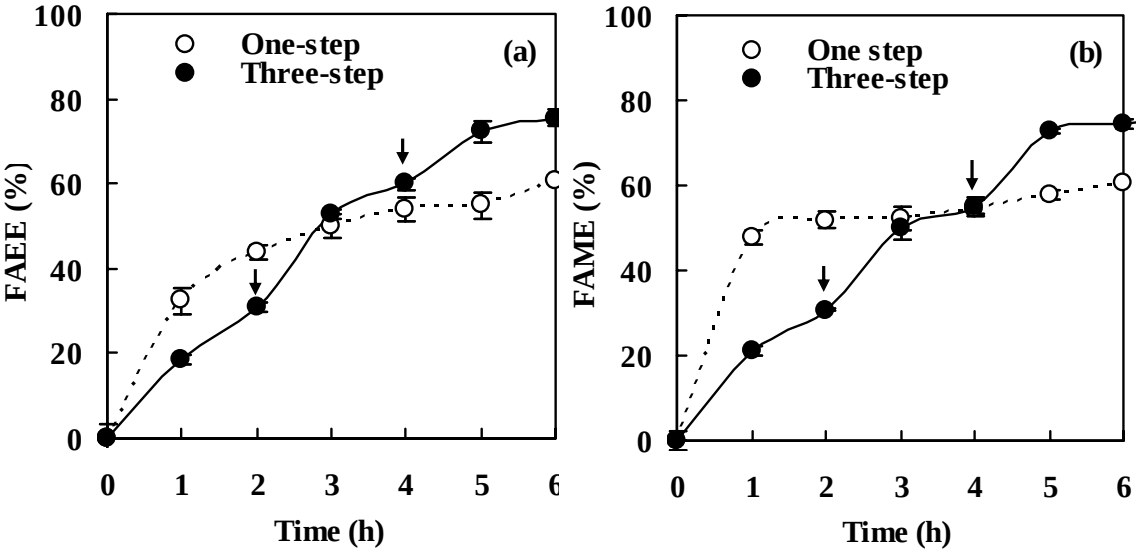

Fig. 6 Effect of three-step addition of ethanol on FAEE production (a) and methanol on FAME production (b) at 0, 2 and 4 h as arrows indicating. Reaction condition; 5 g of palm oil; 50 U of immobilized lipase PS; 0.50 ml of water addition; total 3 molar equivalents of alcohol; and at 45°C.