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Enzymatic Kinetics of Fatty Acids Methyl Ester from Kapok Seed Oil (*Ceiba Petandra*) Using Lipozyme TL IM

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Abstract: Synthesis of Fatty Acids Methyl Ester (FAME) utilizing oil extracted from kapok seeds (*Ceiba petandra*) and catalyzed by lipozyme TL. 1M via enzymatic kinetics transesterification was succesfully conducted A mixture of different FAME, widely known as biodiesel is one of the sources of renewable energy. A kinetic analysis was investigated to identify the reaction model and the rate equation and to calculate kinetic parameters. The result obtained showed that the enzymatic kinetics followed a Michaelis-Menten model. The transesterification kinetic followed the Ping-pong Bi-Bi mechanism characterized by the υ_{max}, $K_{m(KO)}$, $K_{m(Met)}$ values of 3.06×10^{-5} mmol/min.mg catalyst, 0.95×10^{2} ppm, 1.30×10^{2} ppm, respectively.

Keywords: Enzymatic, Kinetics, Fatty Acids Methyl Ester, Kapok Seed Oil

Introduction

FAME could be synthesized through transesterification using several substances, such as animal fats, waste cooking oils or animal fats. The process involving a glyceride and alcohol produces alcohol and fatty acid esters Physically, FAME properties and conventional dieseal are alike. FAME is also famous for its biodegradable and non-toxic nature properties. FAME is widely applied in the manufacture of bio-degradable materials, such as bio-lubricants, bio-fuels and bio-surfactants (Dossat *et al*., 2002; Shahla, 2012).

Some of the raw materials that have been successfully converted into FAME fromseed oil of canola, soybean,palm, sun flower and coconut (Achten *et al*., 2008). However, the raw materials are used as edible oil. One of the sources of non-edible materials that availability is abundant and can be converted into FAME is cotton seed oil. Previous studies showed that the oil content of kapok seed is 30.075%, (Asnawati *et al*., 2014)

The synthesized FAME from the oils can be synthesized using chemicals and enzymatic catalyzed methods (Gunawan *et al*., 2011). The latter method is known to be expensive, however it provides advantages that the former cannot give. The advantage that captivates many researchers is the production of free fatty acids that does not cause saponification in the formed products. Other advantages that are well liked are its ability to produce optimum yield and preferable glycerol recovery. (Calabro *et al*., 2010).

In this work, the enzymatic transesterification of FAME from kapok seeds oil (*Ceiba petandra*) was performed using lipozyme TL. IM the employed lipase is from Thermomyces lanuginosus immobilized on silica gel. The enzim is six time less expensive compared to that of Lipozyme RM (Rhizomucor miehei) (Basri *et al*., 2013). Lipozyme RM IM is commonly used to catalyze the transesterification of oils (Gunawan *et al*., 2004; Awang and May, 2010). The aim of this research is to discover/establish the reaction rate of FAME production. This research imposes a significance contribution in designing a reactor to produce FAME in large quantities as the production process requires reaction kinetics data (Salamah, 2014).

Materials and Methods

Kapok seed oil, Immobilized lipase from Thermomyces lanuginosus produced by Novo Nordisk (Denmark) LA330045, Methanol 98% was obtained from Fluka Chemika (Switzerland), Ester standards: methyl palmitate, methyl stearate, methyl oleate and methyl linoleate were purcashed from Sigma Aldrich (USA), Hexane was purchased from J.T. Baker (USA). All other chemicals were of analytical grade.

Synthesis and Analysis

Different amounts of enzyme were dissolved to 2 ml n-hexane, followed by molar ratios of substrate (oil and methanol). The mixture of Lipozyme TL IM., methanol and kapok seed oil were incubated in a horizontal water bath shaker (120 rpm) at a various reaction time and at temperature of 40°C. Gas chromatograph was used to

analyse the mixture. RTx-65TG capillary column (30 m×0,25 mm, Supelco, USA). Carrier gas employed was helium with flow rate 30 ml/min. The runned temperature was 2 min at 40°C, 8°C/min to 280°C and 5 min at 280°C. Detector used was FID (Flame Ionization Detector) at 300°C.

Kinetic Enzymatic of FAME

The initial rate of the reaction in the presence of different concentration of enzyme and variation of reaction time were studied. The amount of enzyme and reaction time used were 25 to 150 mg MI^{-1} and 6 to 18 h, respectively. Effect of Variation of molar ratio is 1; 0.5 to 0.1; 3.5 mmol (oil; methanol) and the same ratio variation were applied to methanol; oil Water bath shaker (40C/120 rpm) was used to incubate the reaction mixture. Calculation of the initial rate of the reaction was obtained from from the graph of FAME formation against time. The unit of the slope generated from the graph was reported as mmol/min.mg catalyst. The maximum initial rate (V_{max}) and Michaels-Menten constanta (K_m) was calculated using the Lineweaver-Burk plot.

Result and Discussion

Effect of Enzyme Concentration of the Initial Rate

Alcoholysis reaction was used to synthesize FAME using kapok seed oil, methanol and Lipozyme TL 1 M as a catalysis. The Lipozyme was a lipase from *Thermomyces lanuginosus*. The effect of the amount of enzyme on the initial rate could be quantified to determine its catalytic effect. Regioselectivity of lipase plays an important role in specifying triglyceride molecule positions. This gives lipase a specificity properties.

Based on its selectivity, lipase could be categorized into acyl postion (regioselectivity) in the glycerol backbone (Chandler, 2001; Ghaly *et al*., 2010). There are three types of lipase, 1,3 specific, 2 specific, or non specific (Koskinen and Klibanov, 1996). *Thermomyces lanuginosus* wasthe type having a regioselectivity of 1.3 specific lipase (Robles-Medina *et al*., 2009; Ghaly *et al*., 2010).

Figure 1 showed the relationships between theinitial rate of the transesterification of kapok seed oil at different enzyme concentration (25-150 mg L^{-1}) and fixed substrate concentrations. It was found that when the concentration of enzyme rise (25-50 mg L^{-1}), the initial rate increased. This was due to the lipase enzymes that acted as catalyst. The calculated optimum initial rate was 7.412×10^{-4} mmol/min. The presence of the catalyst reduced the activation energy in order to accelerate the occurrence of a reaction. The enzymes were capable to converte fatty acids into FAME completely. However, when the enzyme concentration was 75 to 150 mg mL^{-1}, the initial rate of the reaction decreased and inclined stable, at 4.4×10^{-4} mmol/min. The amount of the enzyme was a significant factor determining the rate and efficiency of the reaction (Manurung *et al*., 2014) Unfortunately, at times, the increase of lipase concentration did not result in higher conversion due to the inhibitor presence. Another reason was because the immobilized enzymes working as the catalyst could no longer react with the substrate, thus preventing the increase of the initial rate of the reaction

Effect of Reaction Time

Figure 2 reveals time course of the enzymatic transesterification Fig. 2 reveals time course of the enzymatic transesterification which could be used as a reference of "enzyme performance and reaction progress It is also an indicator to determine adequate or shortest time needed to achieve expected results, for instance less production expenses and good yields (Yee *et al.* 1997). The percentage yield of FAME showed an increase up to 10 h reaching 80.07% but slightly decreased when it hit 20 h. Reduction of the percentage yield (above 10 h Occurred due to the increase of glycerol to lipase concentration. This limited the surface area contact between the substrate and Lipozyme TL.IM (Basri *et al*.., 2013).

Study of Enzyme Kinetics

Enzyme as a catalyst is one of the attractive areas of study in chemical kinetics. Kinetics study of transesterification of FAME using lipozyme TL. IM. was best achieved by measuring the initial rate in the presence of different substrate concentrations. In enzyme kinetics, it was customary to measure the initial rate of a reaction to minimize reversible reactions and the inhibition of enzymes by products (kinetic book). Figure 3 depicts the relationship of initial rate of transesterification of kapok seed oil in different oil substrate initial concentration (1-3.5 mmol) and fixed methanol concentration.

As observed, the initial rate of the reaction increased with the increase of Methanol (M) concentration and reached the maximum rate at the concentration of 3.5 mmol of Kapok seed Oil (KO)

A similar trend was also found on the use of different kapok seed oil cencentration, where the initial rate of the reaction was also dependent on the methanol concentration.

The presence of enzyme catalysis cause a dramatic increase of the reaction rate and increase high specivity.

In this case, the process involved two substrates. However, the enzymatic catalysis of the two substrates could be controlling concentration of one of the substrates. This results in the formation of a plot following Michelis-Menten relationship between the initial rate and the other substrate concentration (Lai *et al*., 1999). Michaelis and Menten provided an explanation regarding a mechanism of "the initial rate of enzyme-catalyzed reactions" that is highly affected by the concentration. The Curve as shown in Fig. 3 and Fig. 4 was a hyperbolic shape. The hyperbolic indicated that the reactions followed Michaelis-Menten type (Awang *et al*., 2004; Gonze and Kaufman, 2016).

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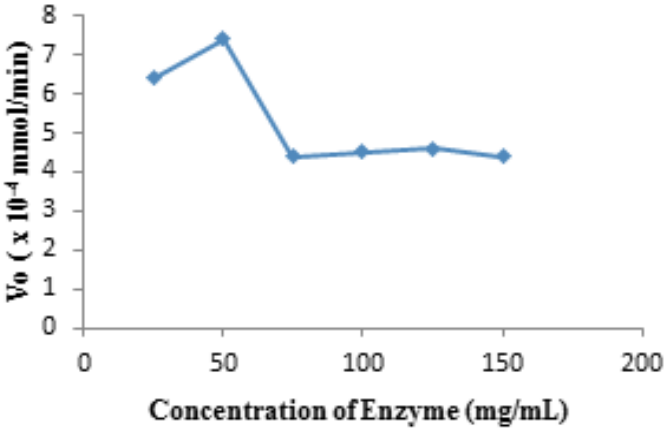


Fig.1. Effect of Enzyme Concentration on the Initial Rate

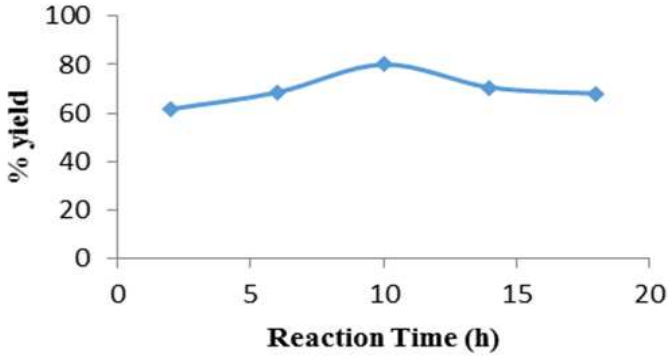


Fig. 2. Effect of reaction time

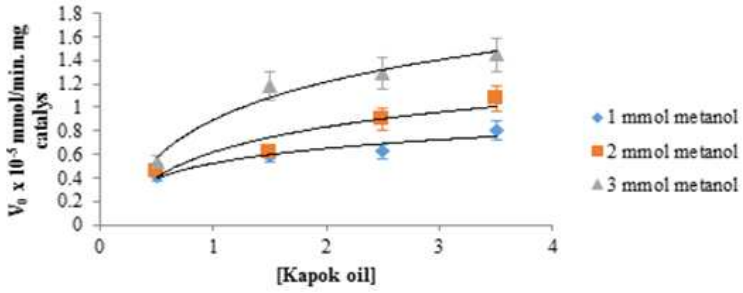


Fig. 3. Initial reaction rate of transesterification as a function of kapok seed oil concentration

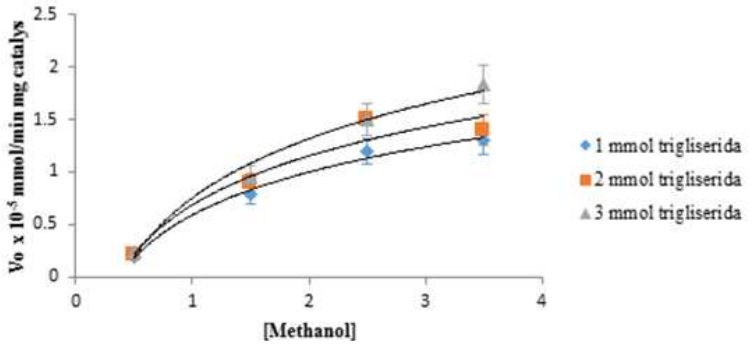


Fig. 4. Initial reaction rate of transesterification as a function of methanol concentration

In an enzymatic reaction, the presence of substrates or other products should be observed as they could act as inhibitors (Romero *et al*., 2007). In this research, alcohol did not show any inhibition activities. The rate equation for this reaction with assumtion no inhibition from both substrates and products was given by Segel (1975) and Romero *et al*. (2007)

$$v = \frac{v_{max}[KO][Met]}{K_{M(KO)}[Met] + K_{M(met)}[KO] + [KO][Met]} \quad (1)$$

where, v_{max} is the maximum reaction rate, v is the initial reaction rate and $K_{M(KO)}$ and $K_{M(met)}$ are the binding constants (Michaelis constants) for both substrates, Kapok Oil (KO) and Methanol (Met). This equation can be modified to linear regression equation.

$$\frac{1}{v} = \frac{K_{M(KO)}[Met] + K_{M(met)}[KO] + [KO][Met]}{v_{max}[KO][Met]} \quad (2)$$

$$\frac{1}{v} = \frac{K_{M(KO)}}{v_{max}[KO]} + \frac{K_M(met)}{v_{max}[Met]} + \frac{1}{v_{max}} \quad 3)$$

For constant methanol concentrations, equation shown below:

$$\frac{1}{v} = \frac{K_{M(KO)}}{v_{max}[KO]} + \frac{1}{v_{max}} \quad (4)$$

And for constant kapok oil concentrations, equation shown below:

$$\frac{1}{v} = \frac{K_{M(met)}}{v_{max}[Met]} + \frac{1}{v_{max}} \quad (5)$$

Figure 5 shows a relationship between the initial rate and concentration of double reciprocal kapok oil at several methanol concentrations. The plot formed follows the double reciprocal line weaver bulk plot, It can be further used to generate the dependency of the intercept values At varied concentrations of methanol to yield the values of K_M for slope and v_{max} for intercept. The values of kinetics constant were shown in Table 1.

The other experiments for reciprocal methanol concentration at several kapok oil concentration were shown in Scheme 1. The experiment with varied kapok oil and methanol concentration. Exhibited that there was a linear correlation between initial reaction rate and substrate concentration studied, in which the initial rate went up when the substrate concentration raised increasd on the linear line as shown in Fig. 6.

The parallel line indicated the reaction mechanism of kapok oil and methanol via enzymatic transesterification followed a Ping-pong Bi-Bi mechanism. The analysis results was used to model the Ping Pong Bi-Bi model and the sequence of the reaction is presented below:

Based on the calculated kinetics parameters (Table 1), it was found Enzyme's affinity leaned more to kapok oil compared to that of methanol since $K_{m(KO)}$ was lower than $K_{m(Met)}$.. Romero *et al*. (2007) reported that in their research that the affinity of enzyme towards the substrate was inversely propotional with the value of Michaelis-Menten constant.

Previous studies have laid a foundation used to understand mechanism of FAME production following a Ping Pong model (Azocar *et al*., 2014; Al-Zuhair *et al*., 2007). The proposed mechanism considered that FAME was mainly produced through transesterification pathway. In Fig. 5 and Fig. 6, it could be observed that the R^2 coefficient of determination of kapok oil and methanol concentration was 0.9897 and 0.9910 respectively. These result indicated that proposed quite well with the experimental data. Similar results were found in Basri *et al*. (2013) and Bhandari *et al*., (2013) studies.

Scheme 2 showed the Enzyme (E) transesterification mechanism for kapok oil enzyme-complex (E-KO) and an acylated enzyme fatty acid complex (E-Ac-G). Glycerol was released as the first product. Afterward, the complex reacted with methanol forming complex an acylated enzyme–methanol (E-Ac-Met). The complex then went through an esterification reaction forming an ester-enzyme complex. The free Enzyme (E) the free enzyme will be regenerated as a second product due to the release of FAME.

Table 1. Kinetics parameter for transesterification of kapok oil and methanol using Lipozyme TL IM

Parameter	Units	Value
υ_{max}	mmol/min.mg catalyst	3.06×10^{-5}
$K_{max\ (KO)}$	ppm	0.95×10^{2}
$K_{max\ (Met)}$	ppm	1.30×10^{2}

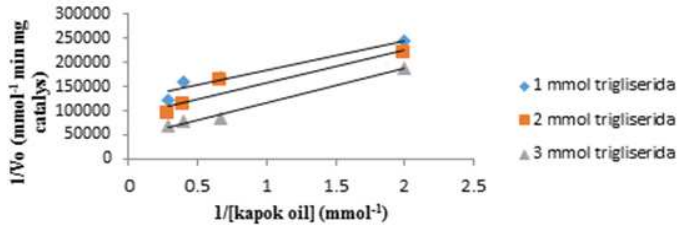


Fig. 5. Line weaver–Burk plots of kapok oil concentration vs initial rate of reaction

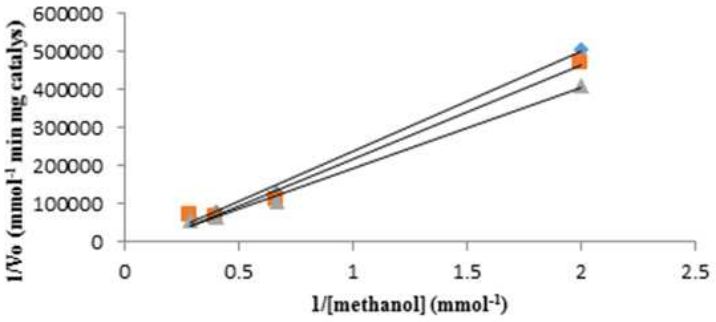
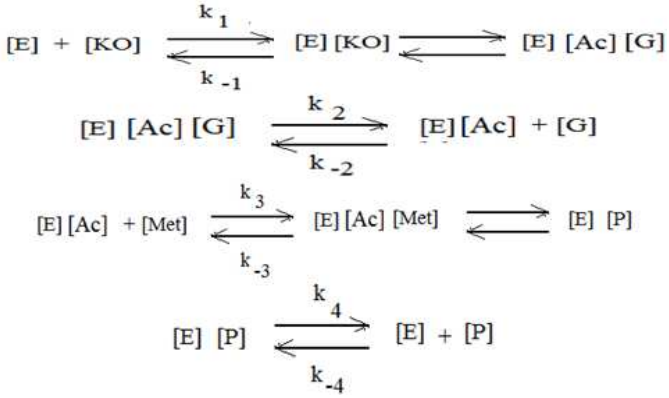
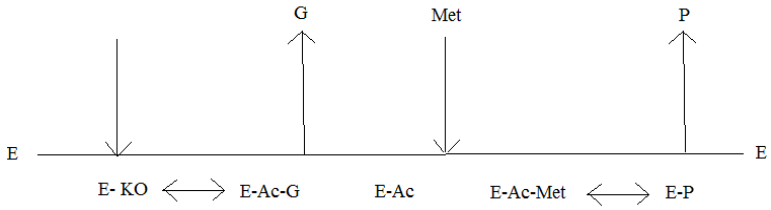


Fig. 6. Line weaver–Burk plots of methanol concentration vs initial rate of reaction



Scheme 1. The Process of Enzymatic reactions, [KO] is kapok oil, [Met] is methanol, [E] is enzyme, [G]=glycerol, [P]=product, [Ac]=acylated, k=rate constanta



Scheme 2. Ping-pong Bi-bi transesterification mechanism of FAME production

A bi-bi Ping-Pong mechanism was also hypothesized to describe the kinetics of enzymatic trans-esterification of glycerides for biodiesel production from triolein and ethanol (Calabro *et al*., 2010).

Conclusion

The kapok seed oil transesterification reaction kinetics with ethanol and Lipozyme TL. 1M was established. The study results showed that the mechanism of the reaction followed the Bi-Bi Ping-Pong mechanism with the absence of inhibition under a concentration of 3,5 mmol for both (Kapok seed oil and methanol) and it followed a-Michaels-Menten kinetics. The affinity of the enzyme leaned more to kapok oil compared to that of methanol because $Km_{(KO)}$ was lower than $Km_{(Met)}$. A parallel line on the graph and the value of R^2 ranging from 0.9897 -0.9910 showed that the models of bi-bi Ping-pong was suitable for this reaction.

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Author's Contributions

Erin Ryantin Gunawan: Participated in all experiments, organized the study and contributed to the writing of the manuscript

Dedy Suhendra: Designed the research plan and coordinated the data-analysis and contributed to the writing of the manuscript

Baiq Rizkia Miftahatul Hasanah: Participated in all experiments and assisted data analysis

Ethics

This article has not been published before and the data listed are in accordance with the results of our team's research. We ensure there is no ethical involvement

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