World Applied Sciences Journal 19 (8): 1105-1111, 2012
ISSN 1818-4952

DOI: 10.5829/idosi.wasj.2012.19.08.2065

Esterification of Glucose Fatty Acids of Coconut Oil Catalyzed by *Candida rugosa* Lipase EC 3.1.1.3 Immobilized on an Indonesia's Natural Zeolite Matrix

Sumi Hudiyono, Sri Handayani and Bali Susilo

Department of Chemistry, Faculty Mathematics and Science,
University of Indonesia, Depok 16424, Indonesia

Abstract: Glucose fatty acid esters can be served as a low calories fat replacer and emulsifier. The study aims to synthesize esters enzymatically catalyzed by immobilized lipase on the activated natural zeolite. Optimal immobilization carried out at temperatures of 37°C, with a ratio of enzyme: zeolite = 1: 3 (w / w) gave 74.81 % loading efficiency. Immobilization occurs on the surface of the zeolite are shown from the results of FESEM analysis with the SE and BSE detectors. Optimum activity of the immobilized enzyme was 35.27 % compared to free lipase. Fatty acid used is hydrolysis products of coconut oil with the largest content of lauric acid (54.10 %). The optimum conditions of esterification occur at temperatures of 40°C, the ratio of glucose: fatty acid 1:60, incubation time of 16 hours and weighs 1.1 g molecular sieve with a maximum product conversion of 8.75%. Degree of esterification product is still low, so that the esterification product is only suitable for emulsifier instead of fat replacer.

Key words: Glucose fatty acid ester · Lipase immobile · Natural zeolite · Loading value · Immobilization efficiency · Optimum esterification

INTRODUCTION

According to the FAO and the FDA, coconut oil can be consumed as a dietary fat that is safe for health. However, excessive consumption can lead to accumulate of cholesterol and other products of metabolism that can lead to coronary heart disease, hypertensions, obesity and even cancer [1]. These negative impacts can be avoided by making the fat has a taste and texture not much different. Simple carbohydrate fatty acid ester has a structure similar to natural oils, is a non-caloric fat substitute that is not digested and not absorbed. Depending on the degree of esterification, fatty acid esters of carbohydrates can serve as an emulsifier at a low degree of esterification or fat replacer in a higher degree of esterification [2, 3].

Carbohydrate fatty acid esters can be prepared by esterification reaction between simple sugars such as glucose to fatty acids derived from coconut oil, both chemically and enzymatically. Most of the production of carbohydrate fatty acid ester is chemically made, but a chemical reaction generally requires a extreme reaction conditions. Esterification reaction using lipase provides distinct advantages, a soft reaction conditions, the low-side products and specific reactions [4]. Some research have been studied in the esterification of fatty acids and a variety of sugar catalyzed by lipase [5, 6].

Lipase (EC. 3.1.1.3) can catalyze various reactions such as hydrolysis, esterification, transesterification (acidolysis, interestericaton, as well as alcoholysis) and aminolysis [7]. In a water medium, the lipase will catalyze the hydrolysis reaction, whereas in organic solvents tend to catalyze esterification and transesterification reactions [8].

Weakness of enzymatic reaction is less stable on changing conditions, availability in small quantities and the difficulty to recover the enzyme from the solution at the end of the reaction. In addition, enzymatic esterification reaction is the reaction equilibrium and produce water that can affect the action of the enzyme lipase so influenced the efficiency of product formation [6]. These barriers can be reduced by immobilized enzyme.

Corresponding Author: Sumi Hudiyono, Department of Chemistry, Faculty Mathematics and Science, University of Indonesia, Depok 16424, Indonesia.

Enzyme immobilization on a matrix of inert and insoluble, are known to have several advantages, among which stabilize the structure of the enzyme thereby increasing resistance to conditions of pH, temperature and organic solvents [9], increases the catalytic activity for esterification reactions and is able to maintain catalytic activity during storage [10].

Organisms that are often used as a source of lipase are *Candida* and *Rhizopus* species. In this study *Candida rugosa* lipase was used. *Candida rugosa* lipase has a molecular mass of 56 kDa with 534 amino acid residues and PI 4.5 [11]. This enzyme works at optimum pH 6.5 to 7.5 and temperatures of 30-35°C [4].

Immobilization by adsorption is the most widely developed for easy and economical, making it suitable to be used for large-scale. Immobilizations of *C. rugosa* lipase by adsorption using a variety of matrices, one silica gel, have been carried out among others by Minovska, *et al*. [12].

MATERIALS AND METHODS

The study is divided into several stages: preparation to hydrolyze fatty acids of coconut oil, lipase immobilized on the zeolite at a temperature variation and the ratio of enzyme to the zeolites, the synthesis of sucrose fatty acid ester. Immobilized enzyme was analyzed by FESEM and the identification of the esterification using FT-IR.

Materials: *Candida rugosa* lipase EC. 3.1.1.3. (PA) from Sigma-Aldrich Corporation, the specific activity of 2.45 U / mg. Commercial coconut oil, purchased at super market in Jakarta. Zeolite is a natural zeolite Klipnotilolith from Bayah, West Java, Indonesia. Molecular sieves type 3A to absorb water.

Coconut Oil Fatty Acids Composition: The results of the analysis of fatty acids in coconut oil by GC of fatty acid methyl ester form (FAME) prepared by standard methods.

Hydrolysis of Palm Oil: Fatty acids derived from coconut oil hydrolysis with 1 M KOH catalyst in alcohol 95 %. Mixture was reflux for 1 h at 62 + 2°C, neutralized with HCl 3 N. Then it extracted with 50 mL of n-hexane twice.

Activation of the Zeolite: Zeolite of 0.8 mm-sized was washed with water and then heated at 70°C for 1 h with repeated three times. Zeolite activated by soaking in a solution of 1 M NaCl for 12 hours with a replacement solution twice. Having removed the water content at a temperature of 105°C, then zeolite calcinated at 300°C for 3 hours. The activated zeolite is then served as a solid phase immobilized lipase.

Immobilized Lipase Preparation: Lipase prepared with the variation of w/w (1:3, 1:4, 1:5) of the zeolite was dissolved in 50 mL Tris-HCl buffer 0.05 M pH 7.0. Zeolites that have been activated put in a buffer solution which already contains lipase. The mixture is stirred with the temperature variation (27, 32, 37 and 45°C) for 1 hour. Expected during the mixing, the zeolite adsorbs lipase to form lipase immobilized. Then, the mixture was filtered with vacuum filter. The filtrate was taken for determination of loading efficiency. Enzyme that has immobilized was dried overnight and stored in the refrigerator until used. Immobilization process was observed by analysis of FESEM (Field Emission Scanning Electron Microscope) using a detector SE (Secondary Electron) and BSE (Electron Back Scattering).

Determination of Immobilized Lipase: Loading efficiency determined indirectly by measuring the amount of enzyme not immobilized by the method of Lowry [13].

Immobilized Lipase Activity Determination: Inserted in erlenmeyer glass container a 0.425 mL coconut oil, 0.5 g arabic gum, added by Tris-HCl buffer pH 7 as much as 7.65 mL and 60 mg immobilized lipase, 1.5 mL (ratio enzyme: zeolite = 1:4) that have been dissolved in buffer pH 7. The mixture was incubated in a horizontal shaker incubator for 1 h at 30°C and 150 rpm. Termination reactions were performed by adding alcohol: acetone = 1:1 as many as 10 mL and then titrated using 0.05 N NaOH to determine the levels of fatty acids released. Triples reactions were performed using a blank form of all the substrates and the same treatment as the samples, without adding the lipase catalyst.

Esterification of Fatty Acids with Glucose: In the container erlenmeyer glass inserted with 0.1 mmol glucose, 6 mmol fatty acids of coconut oil and 0.1 g immobilized lipase. The mixture then added by n-hexane 1:1 v/v and whipped with a horizontal shaker incubator at a 200 rpm at desired temperature [6]. Reactions were performed in triples with a variation in incubation time (4, 8, 16 and 32 hours), temperature (30, 35, 37, 40 and 45°C), molar ratio between glucose and fatty acid (1:12, 1:30, 1:60, 1:90). Weight variation of molecular sieve was added to 0.2 g, 0.7 g, 1.1 g and 1.5 g. Termination

reactions were performed with erlenmeyer soaking in a water bath at a temperature of 80°C. Esterification reaction products were then identified using FTIR.

RESULTS AND DISCUSSION

Coconut Oil Fatty Acids Composition: The analysis results of fatty acids composition in coconut oil gave the following composition: 7.20 % Caprilic (C8: 0), 8.02 % capric (C10: 0), 54.10 % lauric (C12: 0), 17.40 Miristic (C14: 0), 6.64% palmitic (10:0), 1.86% Stearic (C18: 0), 3.99% Oleic (C18: 1), 0.81% Linoleic (C18: 2) and 0.02% linolenic (C18: 3). Average Mr of fatty acid is 207.9.

Triglyceride Hydrolysis of Coconut Oil: Hydrolysis carried out using a solution of KOH in ethanol solvent that serves as an intermediary and help speed up the process of oil hydrolysis. Percent yield of hydrolysis is 92.80 %.

Activation of Natural Zeolites: Natural zeolite Clinoptilolith from Bayah area, West Java, is activated by NaCl to replace Ca^{2+} ions and for harmonizing cations on the structure to neutralize the negative charge of Al-O bond. Calcination at a temperature of 300°C made to evaporate the water trapped in the pores, as well as burning of organic impurities, so that the adsorption of other molecules increases. Characterization of zeolite performed with FTIR as shown in Figure 1. Characterization results show that the spectra are not much different between the natural zeolite with the zeolite activated; the absorption peaks are still similar. This indicates that the activation process does not damage the structure of zeolite. Activation of the zeolite only release impurities and water that still might be trapped in it. As seen in the FT-IR spectra indicating the presence of an OH group (3236-3550 cm^{-1}), Si-OH (2301-2378 cm^{-1}), Si-O (1616-1635 cm^{-1}), Al-O (1041 cm^{-1}) and Na-O (615-617 cm^{-1}).

Lipase Immobilization: Immobilization of enzymes made to add the enzyme more resistance to the environment, such as pH, temperature, solvent, or for the benefit of reuse of the enzyme in a continuous process. Enzyme immobilization on the zeolite is the immobilizing adsorption type [14]. Advantages immobilized using a zeolite is easy to do, inexpensive and easily stored. In Figure 2 (a) presence of the structure of zeolite minerals have absorbed the enzyme, indicated by a red circle. In this section, the topography structure of zeolite has been slightly altered by the presence of enzyme molecules attached, be seen as a bit rough, irregular and as there is debris. This is made clearer at Figure 2 (b), which uses BSE detector; the resulting image can come up with a different contrast between the different chemical compounds. Lighter color in the image defined as compounds that have relatively large molecular weight. The analysis using FESEM showed that lipase was absorbed on the surface of zeolite.

Immobilization Temperature Variation: Figure 3, shows that the optimal temperature of immobilization at 37°C.

Optimization of Enzyme Immobilization by Ratio Variation Zeolite Enzyme: The loading value (%) described by the amount of enzyme adsorbed on a matrix that still have catalytic activity. Efficiency immobilization is the ratio of specific activity of immobilized enzyme with specific activity of the enzyme free.

Figure 4, shows that the greater the ratio (zeolite: enzyme), a growing number of enzymes that immobilized (loading value, %), however the immobilization efficiency decreases. One effect of enzyme immobilization on a matrix is localization of enzyme in the matrix. Localization of these enzymes may have made a greater stability to temperature and pH [10]. But localization also limits the movement of the enzyme. Orientation and conformational changes of enzymes in the matrix is not always in directions that can facilitate the substrate to achieve the catalytic enzyme [14]. As a result, the active enzyme can be closed or blocked so that the enzymatic reaction can not take place.

Esterification of Glucose by Fatty Acids: Esterification is a reversible reaction that influenced by several factors; therefore, need to get the optimum reaction conditions such as temperature reaction, substrate ratio, incubation time and the addition of molecular sieve.

The use of non-polar solvent was chosen so that the lipase tended to catalyze the esterification reaction [7, 15]. As catalysts, enzymes must be within a certain structure and exist in an environment that supports the catalytic activity. Organic solvents that can sustain the activity and stability of the enzyme are solvent with log P values range 2-4. Based on several studies on the esterification reaction with a lipase [16], it is known that organic solvents give the highest conversion percentage is hexane. Hexane is a solvent with a log P value = 3.5 [15]. It is why the n-hexane was chosen as a solvent in this reaction.

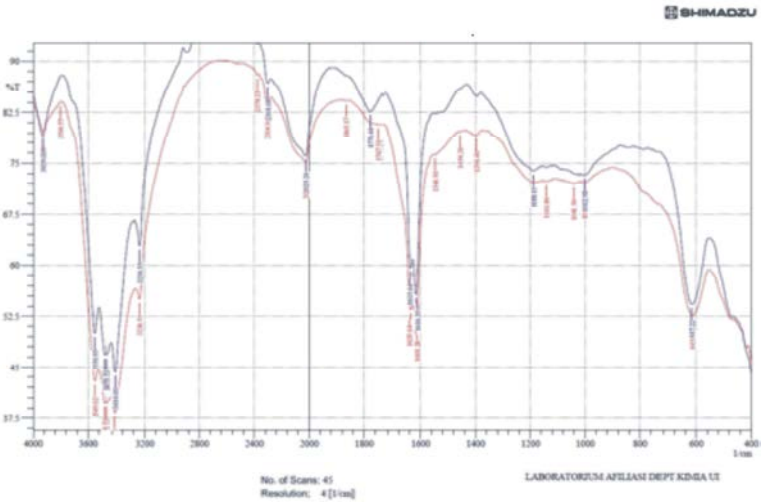

Fig. 1: IR spectra of zeolite before activation (red) and after the activated (blue)

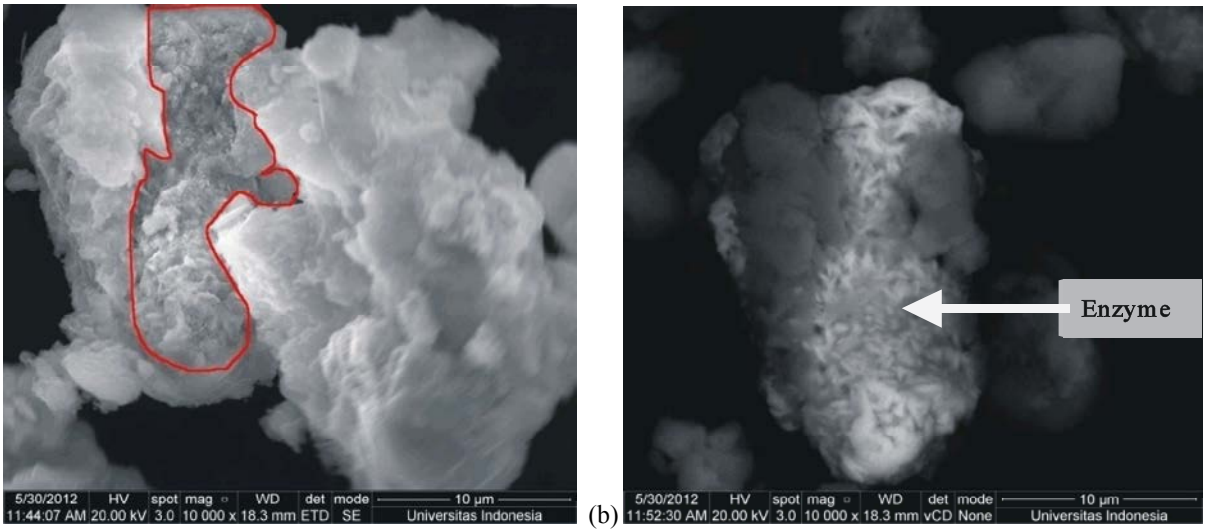

Fig. 2: The Immobilized enzyme on zeolite was analyzed at a magnification of 10,000 times of FESEM using a detector (a) SE (b) BSE

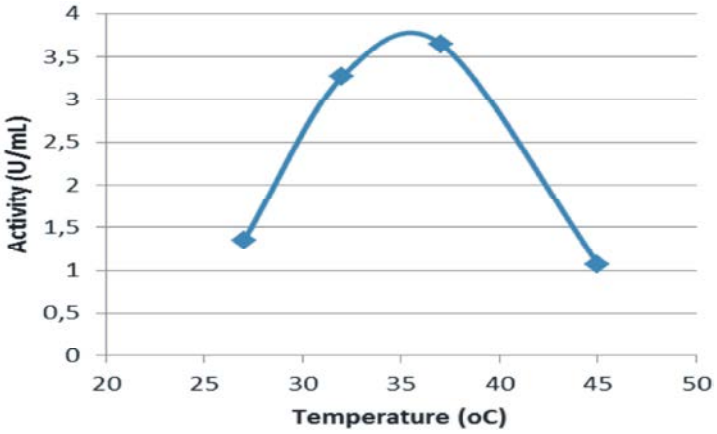

Fig. 3: The effect of temperature on the immobilisation optimations

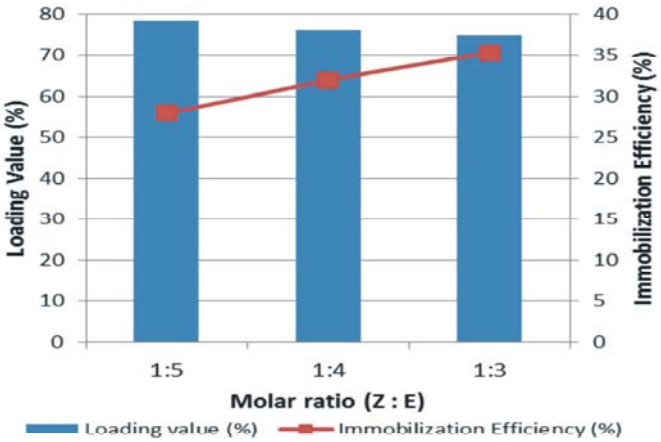

Fig. 4: The effect of ratio of zeolite: enzyme to the loading value (%) and immobilization efficiency (%). Z (zeolite), E (enzyme)

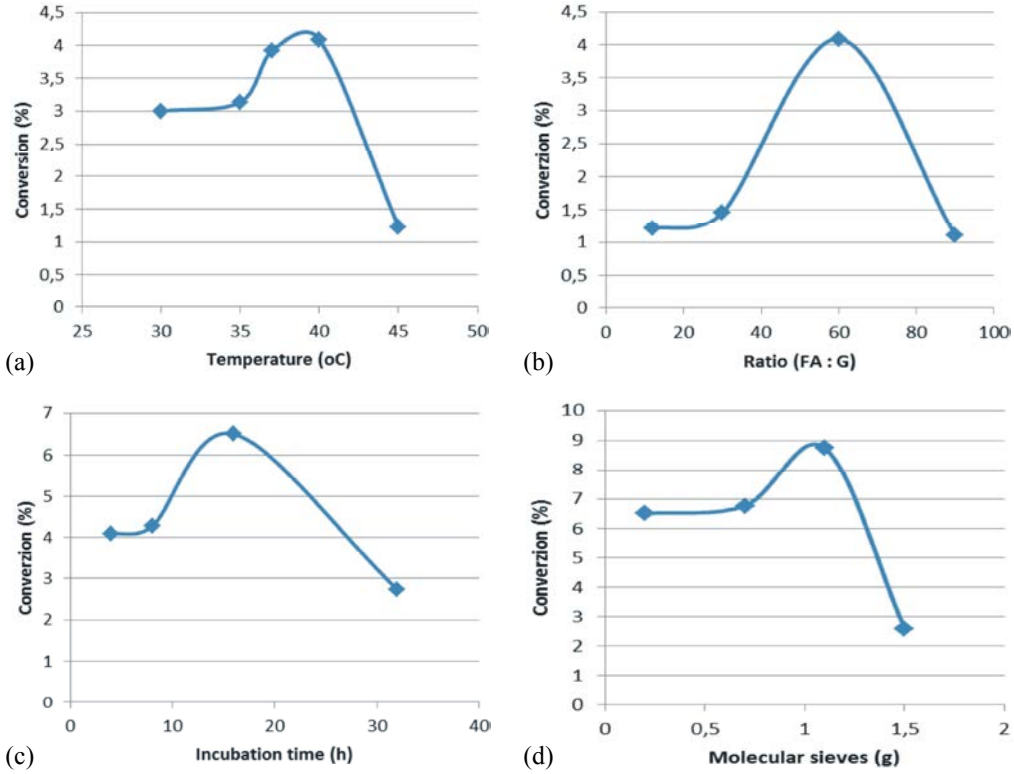

Fig. 5: Optimizing condition for esterification reaction. (a) Temperatures, (b) Ratio glucose: fatty acid (mmol: mmol), (c) Incubation Time (hour) and (d) Molecular sieves added (g)

Optimization of Reaction Conditions: The enzyme requires optimum conditions to obtain the optimal catalytic process. The optimum reaction temperature esterification reaction obtained at 40°C, as shown at Fig. 5a. The ratio of substrates was based on previous studies showing that there is a relationship between the numbers of hydroxyl groups on glucose with the carboxylate groups on the fatty acid [17]. Effect of substrate mole ratio to the % conversion of esterification when the optimal ratio is 1:60 of mmol glucose: mmol fatty acids, Figure 5b.

The higher substrate concentration will increase the reaction rate, until the percent conversions achieve an optimum product. Subsequent increase in substrate

concentration causes a decrease in percent conversion, due to the high concentration of fatty acids that will increase the acidity that would affect the tertiary structure of proteins that cause a decrease in activity.

Incubation time optimization is done by varying the reaction time. Figure 5c, shows that the esterification occurs at the optimal incubation time of 16 hours. The longer reaction occurs due to decrease in percent conversion from the amount of water formed. Excessive water will shift the equilibrium to the reactants and can cause the enzyme tend to catalyze the hydrolysis of ester formed. When the water exceeds the amount needed as essential water, it can lead to mass transfer resistance at the enzyme substrate immobile, so the percent conversion is reduced.

The use of molecular sieve as a pull water meant to draw water products. Figure 5d, shows that the increase in weight of molecular sieve affects on the % conversion. Water is absorbed by molecular sieve to make the equilibrium shifted towards the ester and its conversion products at the optimal weight of 1.1 g. However, the weight of higher molecular sieve, tend to impede the movement of the substrate when is incubated in a horizontal shaker incubator. If contact occurs declining due to lack of movement, then the % conversion products will also be decreased.

Esterification Product Identification: Identification of functional groups by FTIR is shown the peak at 1739 cm^{-1} indicates the presence of the ester group which is still relatively small. This is reinforced by the still large OH peak at 3200-3400 cm^{-1} which shows that not all OH groups of glucose esterified. This is in line with the statement that the fatty acid esters of carbohydrates are synthesized enzymatically difficult to produce with a high degree of substitution [3]. Steric hindrance of the glucose is high; with one primary-OH group is difficult to produce a high degree of substitution. The product is expected to have a low degree of substitution and can act as emulsifiers.

CONCLUSION

Lipase immobilized on zeolite matrix has been successfully performed and showed optimum activity of 35.27 % compared to free lipase activity. An optimum condition of immobilized enzyme was at 37°C and the ratio of enzyme: zeolite = 1:3 (w/w) with 74.81 % loading efficiency. Immobilized lipase can catalyze the esterification reaction between the fatty acids of coconut oil hydrolysis to glucose with the optimum conditions at 40°C, substrate ratio of glucose: fatty acid is 1:60, incubation time of 16 hours and weighs 1.1 g of molecular sieve with the % conversion of the product maximum was only 8.745 %.

Degree of esterification of the product is still low so that the resulting product is only suitable for emulsifier instead of fat replacer.

ACKNOWLEDGMENT

Thank you for the University of Indonesia for financial support of this research.

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